« Nothing in the world can take the place of Persistence.
Talent will not; nothing is more common than unsuccessful men with talent.
Genius will not; unrewarded genius is almost a proverb.
Education will not; the world is full of educated derelicts.
Persistence and determination alone are omnipotent. »

Calvin Coolidge

Cover: *Potential protective roles of MMP-9 in the etiopathogenesis of SLE.*

Katholieke Universiteit Leuven

GROUP BIOMEDICAL SCIENCES
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PROTEOLYSIS OF SYSTEMIC AUTOANTIGENS AND SUPPRESSION OF SYSTEMIC AUTOIMMUNITY BY GELATINASE B/MMP-9

BÉNÉDICTE CAUWE

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DOCTORAL THESIS IN BIOMEDICAL SCIENCES

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ABBREVIATIONS

2D-degradomics	Two-dimensional degradomics
Aβ	β-amyloid protein
Ac-DEVD-CHO	*N*-acetyl-Asp-Glu-Val-Asp-aldehyde
ACR	Albumin-to-creatinine ratio
AEC	Anion exchange chromatography
ALPS	Autoimmune lymphoproliferative syndrome
APMA	*p*-aminophenylmercuric acetate
ARDS	Adult respiratory distress syndrome
B6	C57Bl/6
BAFF	B cell activating factor
BBB	Blood-brain barrier
BCR	B cell receptor
BOOP	Bronchiolitis organizing pneumonia
BSA	Bovine serum albumin
C1q	Complement protein C1q
CAP1	Adenylyl cyclase-associated protein-1
CD	Cluster of differentiation of human (glyco)proteins
CEC	Cation exchange chromatography
CENP-B	Centromere protein-B
CFA	Complete Freund's adjuvant
CRP	C-reactive protein
DAMP	Damage-associated molecular pattern
DN T cells	Double negative T cells
ds/ssDNA	Double-stranded/single-stranded deoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmatic reticulum
F-actin	Filamentous actin
FADD	Fas-associated death domain-containing protein
FDC	Follicular dendritic cell
G-actin	Globular actin
Gld	Generalized lymphoproliferative disease
GST	Glutathione-*S*-transferase
HMGB1/2	High mobility group box 1/2 protein
HSP	Heat shock protein
IC	Immune complex
ICAM-1	Intercellular adhesion molecule-1;
ICM	Intracellular matrix
IEC	Ion exchange chromatography
IFN-α/β	Interferon-α/β
Ig	Immunoglobulin

Interleukin-	IL-
kDa	Kilodalton
-L	Ligand
LC-MS/MS	Liquid chromatography tandem mass spectrometry
Lpr	Lymphoproliferative
mDC	Mature dendritic cell
MFG-E8	Milk-fat globule epidermal growth factor (EGF)-factor
MHC II	Major histocompatibility complex Class II
MMP	Matrix metalloproteinase
MS	Mass spectrometry
MT-MMP	Membrane-type MMP
NEC	Necrotic cell-derived material
NGAL	Neutrophil gelatinase B-associated lipocalin
NPSLE	Neuropsychiatric systemic lupus erythematosus
O-phen	1,10-phenantroline
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate-buffered saline
pDC	Plasmacytoid dendritic cell
pI	Isoelectric point
PVDF	Polyvinylidene fluoride
-R	-receptor
RAGE	Receptor for advanced glycation end-products
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sylphate-polyacrylamide gel electrophoresis
SLE	Systemic lupus erythematosus
SNEC	Secondary necrotic cell-derived material
snRNP	Small nuclear ribonucleic protein
SSA/B	Sjögren's syndrome antigen A/B
TCR	T cell receptor
TGF-β	Transforming growth factor-β
TIMP	Tissue inhibitor of metalloproteinases
TLR	Toll-like receptor
TNF	Tumor necrosis factor
T_{reg}	Regulatory T cell
VCAM-1	Vascular cell adhesion molecule-1
Z-VAD.fmk	Z-Val-Ala-Asp.fluoromethylketone

INTRODUCTION

Autoimmune diseases cumulatively affect 5% to 10% of the general population and are a significant cause of morbidity and mortality worldwide. The economic burden of autoimmune diseases is enormous. For example, the National Institutes of Health estimate that over 23 million United States residents have an autoimmune disease, which accounts for yearly direct costs of approximately $100 billion. As a comparison, direct costs related to cancer and vascular diseases are $57 and $200 billion, respectively [1]. At the level of the individual patient, autoimmune diseases often have a negative impact on quality of life and are associated with high healthcare costs and significant productivity loss [2-5]. These socioeconomic facts reflect the complexity of autoimmunity and the often mysterious etiologies of autoimmune diseases, which are the result of immune responses against antigens of the host (self-antigens). Indeed, autoimmunity is very seldom set off by a single cause but is triggered by a variety of agents and dysfunction of multiple molecular and cellular pathways. As with a genetic predisposition to cancer, inherited defects in self-tolerance genes typically precipitate autoimmune diseases stochastically after a latent phase. Multiple mutations, both inherited and somatic, may be required for a self-reactive clone to circumvent sequential tolerance checkpoints. In addition, environmental factors such as toxins, viral infections and traumatic injury may synergize with accumulated mutations to tip the balance from tolerance to autoimmunity [6,7].

In organ-specific autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, ulcerative colitis and type I diabetes, the autoantigens are mostly confined to the affected organ. In systemic autoimmunity, however, the autoantigens typically are ubiquitous intracellular proteins. A prototypic systemic autoimmune inflammatory condition is systemic lupus erythematosus or SLE, in which genetic, epigenetic, hormonal and environmental factors result in the dysregulation of all the key components of the immune system, culminating in the production of pathogenic autoantibodies against intracellular and mostly intranuclear proteins and nucleic acids [8,9]. The formation and deposition of immune complexes (ICs) in various tissues result in the activation of complement and ultimately lead to acute and chronic inflammation. Consistent with the systemic nature of SLE autoantigens, auto-immune tissue injury can extend to nearly any organ system and the common clinical presentations of SLE range from fatigue, rash and arthritis, through anemia and thrombocytopenia to serositis, nephritis, seizures and psychosis, with fluctuating intensity and severity within and between patients [10,11].

World-wide, prevalences of SLE range from 20 to 70 per 100 000, whereas incidence rates vary from approximately 1 to 10 per 100 000 person-years [12]. Since around 90% of SLE patients are women, gender is obviously the strongest risk factor for SLE. An increased risk among reproductive age women is clearly seen in African Americans, whereas in other populations the highest age-specific incidence rates occur in women after the age of 40 years. Disease frequency and severity are two to four times higher among nonwhite populations around the world, and disease tends to be more severe in men and in pediatric and late-onset SLE. The life expectancy of SLE patients has improved from a survival of less than 50% at 5 years (after diagnosis) in the 1950s

to a current survival of 85% at 10 years and 75% at 20 years. Hence, SLE is characterized by a bimodal pattern of mortality, with early mortality mainly caused by SLE pathology or by immunodepression-induced infections, whereas patients with SLE who live longer develop new disease- and therapy-induced comorbidities such as accumulation of organ damage, osteonecrosis and coronary heart disease [11,13]. A less favorable survival prognosis is often observed for ethnic minorities, which is possibly partly related to socioeconomic status rather than to ethnicity *per se*, and adequate social support has been shown to be a general protective factor in SLE patients [12].

SLE is a polygenic disease and the genetic influences on SLE development generally result from the combined effects of a large number of genes, with each allele contributing only mildly and the requirement of accumulation of several genes to contribute significantly to an increased risk of SLE [14-17]. However, in some rare cases (e.g. C1q), disease may be caused by deficiencies in a single gene [18]. Besides the genetic component, the contribution of environmental factors to the generation of additional immune dysfunctions in SLE is undeniable. Indeed, the concordance rate for SLE is only 24-57% among monozygotic twins and approximately 2-5% among dizygotic twins [14]. Epigenetic changes such as the observed DNA hypomethylation in SLE T and B cells may be caused by drugs such as hydralazine and procainamide. Exposure to ultraviolet light is a known risk factor for relapses in clinical disease, and various environmental toxins, such as silica, pesticides, mercury and smoking may increase the risk of SLE [10,11]. Finally, viral infection may trigger systemic autoimmunity by the induction of type I interferons and by molecular mimicry, which is the cross-reaction of T cells, B cells or antibodies against viral, bacterial or parasitic epitopes with self-antigens [19,20].

Despite the identification of multiple genetic and environmental risk factors, the etiology and pathogenesis of SLE are incompletely understood and current therapies rely largely on the use of immunosuppressive corticosteroids and cytotoxic anti-proliferative drugs, which have limited efficacy and carry significant risks of toxicity and development of the above-mentioned comorbidities. During the past decade, however, studies of the key components of the immune system in multiple animal models of SLE and in patients have provided important new insights into underlying disease mechanisms and have led to an etiopathogenesis model in which aberrant clearance of and immune reaction to apoptotic and necrotic debris play a central role in the breaking of systemic tolerance and promotion of autoimmune tissue injury (Figure 1) [21-27].

Apoptosis, the most common type of programmed cell death, plays a pivotal role in embryogenesis, development and homeostasis of multicellular organisms [28]. In higher organisms, billions of apoptotic cells generated during the maintenance of homeostasis are rapidly and efficiently removed by professional phagocytes in an immunologicaly silent way [29]. Apoptotic cells actively secrete ‘find me’ signals (e.g. lysophosphatidylcholine (LPC), nucleotides, apoptotic blebs), which stimulate a chemotactic response in macrophages, but repel neutrophils [27,29]. Macrophages identify their prey by ‘eat me’ signals such as externalized phosphatidylserine, which is bound by engulfment receptors (e.g. Mer tyrosine kinase, CD14) or indirectly by bridging molecules (e.g. milk-fat globule epidermal growth factor (EGF)-factor

(MFG-E8), growth arrest-specific 6 (Gas6)), triggering the internalization of the apoptotic cell. Upon ingestion of the apoptotic prey, macrophages produce anti-inflammatory 'tolerate me' cytokines, including transforming growth factor-β (TGF-β) and interleukin (IL)-10 [27,29].

If this efficient clearance process fails, apoptotic cells will progress to secondary necrosis, release pro-inflammatory danger signals and lose their membrane integrity with consequent spilling of the intracellular contents into the extracellular milieu (*cf.* Figure 1). In SLE patients, many defects were found with respect to the clearance of apoptotic cells and necrotic cell remnants, such as decreased C-reactive protein (CRP) levels, complement deficiencies, reduced serum DNase I activity and various phagocytic defects [18,23,27,30]. In addition, lymphocytes of SLE patients show increased apoptosis rates [22,30-32]. Hence, upon exposure to apoptotic triggers such as ultraviolet light, toxins, drugs, trauma and infections, an overload of circulating apoptotic cells and uncleared secondary necrotic material will be generated in SLE patients.

These apoptotic and necrotic debris, also called secondary necrotic cell-derived material or SNEC, accumulate in germinal centers of SLE patients, where their uptake by tingible body macrophages is impaired, and associate with follicular dendritic cells (FDCs) [33]. Presentation of SNEC by FDCs to autoreactive B cells that were generated during the random process of somatic mutation may provide survival signals for these self-reactive cells that normally undergo apoptosis and rapid removal by tingible body macrophages. The positively selected SNEC-reactive B cells leave the germinal centre and migrate into the mantle zone where they may receive further costimulation by interaction with $CD4^+$ T cells and differentiate into autoantibody-secreting plasma cells or self-specific memory cells (*cf.* Figure 1) [23,27]. Alternatively, these autoreactive B cells may encounter their cognate autoantigen bound to DNA or RNA (e.g. histones, small nuclear ribonucleic particles (snRNPs) such as U1snRNP and Smith antigen, Ro/Sjögren's syndrome antigen A (SSA), La/SSB). The first activation signal is then provided by binding and internalization of the autoantigen by the B cell receptor (BCR). In the endosomal compartment DNA may interact with toll-like receptor-9 (TLR9) and RNA with TLR7, producing the second activation signal for the autoreactive B cell and leading to autoantibody secretion [34-36].

Under homeostatic conditions, the removal of apoptotic cells by macrophages and dendritic cells is tolerogenic. However, in the presence of danger signals (e.g. high mobility group box 1 protein (HMGB1) and uric acid) released by necrotic cells in SLE, dendritic cells may become activated instead of tolerogenic. Activated DCs then produce increased amounts of pro-inflammatory cytokines (e.g. IL-6 and interferon-α (IFN-α) and break tolerance by the immunogenic (instead of tolerogenic) presentation of SNEC to autoreactive T cells (*cf.* Figure 1) [37,38]. Activated autoreactive T cells will then provide help to autoreactive B cells, which will also result in the production of autoantibodies.

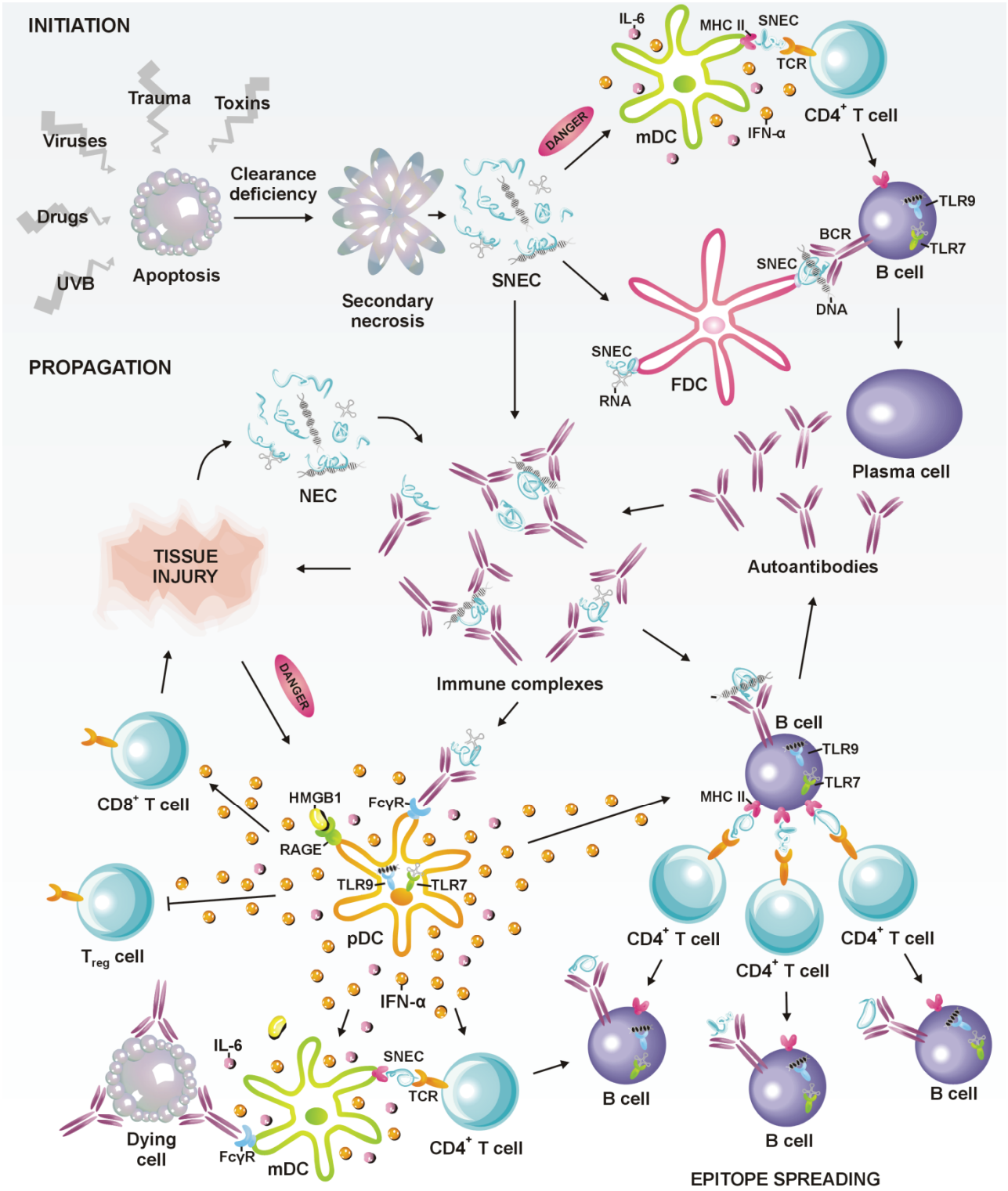

Figure 1. Etiopathogenesis model of SLE. Enhanced apoptosis rates and defective clearance of apoptotic cells are central deficiencies in SLE. Hence, upon exposure to apoptotic triggers such as ultraviolet light (UVB), toxins, drugs, trauma and viral infections, an overload of circulating apoptotic cells and uncleared secondary necrotic cell-derived material (SNEC) accumulates in germinal centers of SLE patients. Follicular dendritic cells (FDCs) present SNEC to autoreactive B cells, which differentiate into autoantibody-secreting plasma cells after costimulation by autoreactive $CD4^+$ T cells. Alternatively, autoreactive B cells may encounter their cognate autoantigen bound to nucleic acid and become activated by binding and internalization of the autoantigen by the B cell receptor (BCR), and interaction of the bound DNA/RNA with toll-like receptor-9 (TLR9)/TLR7 in the endosomal compartment. The presence of danger signals released by necrotic cells leads to secretion of IL-6 and IFN-α by mature dendritic cells (mDCs) and immunogenic presentation of SNEC to autoreactive T cells, which subsequently provide help to autoreactive B cells, resulting in autoantibody production.

After the initiation phase, in which tolerance to self is broken by the above-mentioned mechanisms, systemic autoimmunity will be further propagated by the clearance deficiency in the periphery. Indeed, secreted SNEC-reactive autoantibodies will encounter autoantigens that are continuously supplied by the clearance deficiency in the periphery and form immune complexes (ICs). Deposition of ICs in the tissues causes direct inflammation and tissue injury, which result in additional supply of necrotic material (NEC). Alternatively, IC may activate plasmacytoid dendritic cells (pDCs) by their binding and uptake via Fcγ receptor(R)IIa and concomitant interaction of HMGB1 with the receptor for advanced glycation end-products (RAGE) or other innate immune receptors. Intracellular activation of TLR7 and TLR9 by nucleic acid-containing ICs, results in massive production of IFN-α [34,35], which was recently discovered to play central roles in SLE. IFN-α induces increased expression of autoantigens, maturation and activation of DCs, with increased expression of costimulatory molecules, chemokine receptors and the B lymphocyte stimulator (Blys)/B cell activating factor (BAFF), which promotes efficient autoantigen presentation to $CD4^+$ T helper cells. Furthermore, IFN-α stimulates cytotoxic $CD8^+$ T cells and impairs regulatory T cell (T_{reg}) function in SLE, leading to additional tissue injury. IFN-α stimulates B cell activation, differentiation, antibody production and immunoglobulin (Ig) isotype switching. Hence, the increased IFN-α levels in SLE patients result in a potent amplification of systemic autoimmune reactions [39].

Figure 1. ***Continued.*** After tolerance to self is broken in the initiation phase, systemic autoimmune responses are further propagated by the clearance deficiency in the periphery, which produces a continuous supply of SNEC. Deposition of ICs in the tissues causes inflammation and tissue injury, which result in additional supply of necrotic material (NEC). Alternatively, ICs may activate plasmacytoid dendritic cells (pDCs) by their binding and uptake via Fcγ receptor(R)IIa and concomitant interaction of the danger signal high mobility group box 1 protein (HMGB1) with the receptor for advanced glycation end-products (RAGE) or other innate immune receptors. Intracellular activation of TLR7 and TLR9 by nucleic acid-containing ICs, results in massive production of IFN-α, which induces maturation and activation of DCs and efficient autoantigen presentation to $CD4^+$ T helper cells. IFN-α also stimulates cytotoxic $CD8^+$ T cells and impairs regulatory T cell (T_{reg}) function in SLE, leading to additional tissue injury. IFN-α stimulates B cell activation, differentiation, antibody production and immunoglobulin (Ig) isotype switching. Alternatively, anti-nucleic acid ICs may directly activate autoreactive B cells by BCR/TLR coengagement, which leads to B cell proliferation, differentiation and Ig class switching independently of T cell help. Since a single B cell can present different autoantigen epitopes to T cells, various autoreactive T cells may be activated, which in turn promote the activation of additional autoreactive B cells, leading to epitope spreading. FcγR-mediated phagocytosis of autoantibody-coated dying cells results in the precipitation of inflammation, which further contributes to chronic inflammation and multiple organ damage in SLE. IL-6, interleukin-6; IFN-α, interferon-α; MHC II, major histocompatibility complex Class II; TCR, T cell receptor.

As discussed above, anti-nucleic acid ICs may directly activate autoreactive B cells by BCR/TLR coengagement, which leads to B cell proliferation, differentiation and Ig class switching independently of T cell help [34-36]. Besides their role in autoantibody production, B cells also have multiple autoantibody-independent roles in SLE. B cells can efficiently present antigen and activate T cells, they can augment T cell activation through costimulatory interactions and they can produce many cytokines that affect inflammation, lymphogenesis and immune regulation [40]. In addition, since a single B cell can present different autoantigen epitopes to T cells, various autoreactive T cells may be activated, which in turn promote the activation of additional autoreactive B cells, leading to epitope spreading (*cf.* Figure 1) [36]. An additional pathogenic effect of autoantibodies lies in the opsonization of dying cells. Whereas clearance of apoptotic cells by engulfment receptors is considered a neutral or even anti-inflammatory process, favoring the induction of tolerance, FcγR-mediated phagocytosis of autoantibody-coated dying cells results in the precipitation of inflammation, which further contributes to the chronicity and multiple organ damage of SLE [27,41].

The clearance hypothesis in SLE was nicely confirmed by murine studies, in which genetic deficiency of various molecules that promote the clearance of apoptotic remnants induced systemic autoimmunity (e.g. C1q, DNase I, serum amyloid protein (SAP), Mer, MFG-E8) [16,17]. However, other mouse models with defective removal of dying cells (e.g. mannose binding lectin (MBL)- and CD14-knockout mice) failed to develop autoimmunity, suggesting that compensatory mechanisms may exist in healthy individuals [27]. Hence, the amount of self-antigen may only be sufficient to drive systemic autoimmunity on a susceptible genetic background. Indeed, severe tissue trauma, which also results in an overload of intracellular debris, only triggers autoimmunity in mice and patients with a certain genetic predisposition [42,43]. Studies in mice and men have shown that SLE is associated with dysfunctions throughout the entire immune system, as well as epigenetic alterations, which may act synergistically with the described clearance deficiencies to induce a full-blown autoreactive response [15-17,24]. For example, in healthy persons the immunogenicity of self-DNA is minimized by CpG suppression, CpG methylation, and inhibitory motifs that act together with the inaccessibility to TLRs, which are sequestered within the endosomal compartment. In SLE, the immunogenicity of self-DNA/RNA is not only enhanced by the increased release from necrotic cells, but also by a higher frequency of CpG dinucleotides, as well as by hypomethylation and oxidative modification [35]. In addition, IFN-α may decrease the selectivity of B cells for CpG-rich DNA and allow stimulation by even non-CpG DNA [44]. Hyperactivated SLE T cells provide excessive help to B cells and mount inflammatory responses, while failing to produce sufficient IL-2. This is caused by various defects in signal transduction (e.g. diminished expression of the CD3ζ chain), by abnormal gene transcription (e.g. increased expression of the costimulatory molecule CD40 ligand (CD40L) and the adhesion molecule CD44) and by hypomethylation of T cell DNA. Decreased IL-2 production may contribute to the defective T_{reg} function, the defective $CD8^+$ T cell cytotoxic capacity and the decreased activation-induced cell death in SLE patients [15]. Patients with SLE contain abnormally high levels of serum IL-17, which together with BAFF controls the survival and proliferation of B cells and their differentiation into Ig-secreting cells [45]. A high fraction of $CD4^+$ and $CD3^+B220^+CD4^-CD8^-$ double negative

(DN) T cells in these patients produce IL-17 [46,47]. DN T cells only represent 1% to 2% of peripheral T lymphocytes in healthy persons [48], but are expanded in the peripheral blood of SLE patients. In addition, SLE DN T cells are characterized by increased levels of activation markers [49], production of IL-4 [50], and induction of Ig and pathogenic anti-DNA autoantibodies [51]. IL-17-producing DN T cells were found in kidney biopsies of patients with lupus nephritis and were shown to induce nephritis in a non-autoimmune mouse model [46,52]. Similar to T cells, a marked increase of hyperactivated and memory B cells is observed in SLE [24,25]. Finally, post-translational modifications such as phosphorylation, transglutamination, deamidation, methylation and caspase- and granzyme B-mediated proteolysis were proposed to generate neo-epitopes that may initiate or exacerbate systemic autoimmunity [53-55].

In conclusion, SLE is a complex multifactorial disease, in which multiple clearance deficiencies and immune system aberrations accumulate and synergize in the breaking of systemic immune tolerance and in the amplification of systemic autoimmune responses, culminating in multisystem tissue injury and end-organ damage.

Matrix metalloproteinases are Zn^{2+}-dependent endopeptidases with homeostatic roles in development, reproduction and wound healing. However, dysregulation of MMP activity contributes to multiple pathologic conditions such as cancer, vascular and neurodegenerative diseases, uncontrolled inflammation and autoimmune diseases [56-60]. Gelatinase B/MMP-9 is mainly produced by neutrophils and macrophages during inflammation and may influence immune function by the proteolysis of cytokines and chemokines [61,62]. In addition, MMP-9 releases immunodominant T cell epitopes from myelin basic protein and αB-crystallin in multiple sclerosis [63,64], from collagen type II in rheumatoid arthritis [65] and from insulin in type I diabetes [66], which may explain why MMP-9-deficient mice are less susceptible to develop disease symptoms in these organ-specific autoimmune diseases [67-70]. The integration of balances between pro- and anti-inflammatory cytokines, and between proteases and protease inhibitors in the generation of remnant epitopes for T cells was formulated as the REGA (**R**emnant **E**pitopes **G**enerate **A**utoimmunity) model for autoimmunity [69]. This model explained how proteinase-generated linear peptide epitopes contribute to activation of T lymphocytes.

Hence, the parallel was drawn to a possible role of MMP-9 in the modulation of systemic autoantigens. On the one hand, MMP-9 may play an exacerbating role by the release of neo-epitopes that stimulate autoreactive T cells, as was observed in organ-specific autoimmune diseases. On the other hand, MMP-9 may contribute to extracellular (or intracellular) degradation of autoantigens during apoptosis and secondary necrosis, and have protective effects by the destruction of immunogenic epitopes for T and B cells. In the latter case, lack of MMP-9-mediated proteolysis would engender an additional clearance defect in SLE. In addition, MMP-9 deficiency was shown to enhance proliferation of alloreactive T cells [71,72], suggesting that MMP-9 may play a role in lymphocyte homeostasis. Hence, investigating the influence of MMP-9 in a disorder with dysregulated adaptive immunity, such as SLE, may yield novel insights on the role of matrix metalloproteinases in lymphocyte homeostasis.

AIMS

The primary objectives of the present doctoral research were to evaluate the roles of MMP-9 in the **proteolysis of systemic autoantigens** and in the **development of systemic autoimmunity**. To this end, three main research paths were followed, which are summarized below.

Characterization of the physiopathological roles of MMPs in cell surface proteolysis

MMPs were initially identified as extracellular matrix (ECM)-consuming enzymes [73]. However, soon thereafter compelling evidence emerged showing that these proteases not only degrade structural molecules but also a whole assortment of soluble proteins with very diverse biological functions [56,74,75]. On the route from extracellular to intracellular substrates, an evident intermediate station was the analysis of MMP substrates at the boundary between the extracellular and intracellular milieu. Since a comprehensive (re)view on the cleavage of cell surface-associated substrates by MMPs was lacking, the first aim of this doctoral research was to generate a systematic survey of all membrane-associated MMP substrates, or more specifically of all proteins tethered to the cell surface by a transmembrane domain that were described to be modified by MMPs in a particular physiopathological context (Chapter 1).

Systematic identification of intracellular proteins as substrates for MMP(-9)

In order to define whether MMPs, and MMP-9 in particular, were capable of modulating (intracellular) systemic autoantigens, a systematic search for intracellular MMP-9 substrates was undertaken using a one-dimensional degradomics approach (Chapter 2) and by developing and applying multidimensional degradomics methods (Chapter 3). By cataloguing the biological functions of identified intracellular candidate substrates, an overview of the intracellular MMP-9 degradome was generated to clarify potential roles of MMPs in intracellular protein proteolysis in general, and in systemic autoantigen cleavage in particular.

Characterization of the role(s) of MMP-9 in the development of systemic autoimmunity *in vivo*

Since little was known on the functions of MMP-9 in systemic autoimmune models, mice were generated with both MMP-9 deficiency and the *lpr* (lymphoproliferative) loss-of-function mutation in the apoptosis-inducing receptor Fas on a C57Bl/6 background (Chapter 4). C57Bl/6 mice lacking functional Fas develop moderate lymphoproliferation and late-onset systemic autoimmunity. Hence, besides the influence of MMP-9 on systemic autoimmunity, this model permitted to study the role of MMP-9 in the (dys)regulation of lymphocyte homeostasis.

ARTICLES

CHAPTER 1. THE BIOCHEMICAL, BIOLOGICAL, AND PATHOLOGICAL KALEIDOSCOPE OF CELL SURFACE SUBSTRATES PROCESSED BY MATRIX METALLOPROTEINASES

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The Biochemical, Biological, and Pathological Kaleidoscope of Cell Surface Substrates Processed by Matrix Metalloproteinases

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ABSTRACT Matrix metalloproteinases (MMPs) constitute a family of more than 20 endopeptidases. Identification of specific matrix and non-matrix components as MMP substrates showed that, aside from their initial role as extracellular matrix modifiers, MMPs play significant roles in highly complex processes such as the regulation of cell behavior, cell-cell communication, and tumor progression. Thanks to the comprehensive examination of the expanded MMP action radius, the initial view of proteases acting in the soluble phase has evolved into a kaleidoscope of proteolytic reactions connected to the cell surface. Important classes of cell surface molecules include adhesion molecules, mediators of apoptosis, receptors, chemokines, cytokines, growth factors, proteases, intercellular junction proteins, and structural molecules. Proteolysis of cell surface proteins by MMPs may have extremely diverse biological implications, ranging from maturation and activation, to inactivation or degradation of substrates. In this way, modification of membrane-associated proteins by MMPs is crucial for communication between cells and the extracellular milieu, and determines cell fate and the integrity of tissues. Hence, insights into the processing of cell surface proteins by MMPs and the concomitant effects on physiological processes as well as on disease onset and evolution, leads the way to innovative therapeutic approaches for cancer, as well as degenerative and inflammatory diseases.

KEYWORDS ectodomain shedding, receptor, membrane, cancer, immunity, autoimmunity

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INTRODUCTION

Matrix metalloproteinases (MMPs) constitute a family of over 20 different endopeptidases characterized by a conserved Zn^{2+}-binding motif **H**EXX**H**XXGXX**H** in the catalytic domain and a number of conserved protein domains. The three histidines in this motif are responsible for binding the catalytic Zn^{2+} ion (Figure 1) (Nagase and Woessner, 1999; Brinckerhoff and Matrisian, 2002). Expression of most MMPs is under transcriptional regulation, e.g., by growth factors, hormones, cytokines and oncogenic transformation. MMPs are neutral endopeptidases produced as secreted or membrane-bound pro-enzymes or zymogens, which become activated by removal of the NH_2-terminal propeptide. The interaction of a conserved cysteine in the propeptide with the catalytic Zn^{2+} ion seals the catalytic site and results in the latency of the pro-enzyme (Figure 1) (Visse and Nagase, 2003). Removal of the propeptide, for example by proteolysis, alters this coordination with Zn^{2+}. Due to the subsequent conformational change, the Zn^{2+} ion becomes available for the binding of a hydrolytic water molecule and of the substrate. Therefore, the MMP activation mechanism was named the 'cysteine switch mechanism' (Van Wart and Birkedal-Hansen, 1990). This activation can be mediated by proteases and other MMPs, or chemically by means of organomercurials, urea, some detergents and also by reactive oxygen species. In addition, some MMPs are activated intracellularly by furins (Nagase and Woessner, 1999).

Examples of 'archetypical' MMPs are the collagenases, which are active against fibrillar collagens, and the stromelysins, which cleave non-collagen components of the extracellular matrix (ECM) (Folgueras *et al.*, 2004). They contain a COOH-terminal hemopexin domain (Figure 1), which contributes to substrate specificity, and to interactions with endogenous inhibitors and cargo receptors (Piccard *et al.*, 2007). This hemopexin domain is absent in the smallest MMPs, the matrilysins. In addition, the gelatinases, active on denaturated collagens, incorporate three fibronectin type II repeats for the binding of gelatin, and MMP-9 is the only MMP to possess a Ser/Thr/Pro-rich O-glycosylated domain, which forms an attachment site for multiple O-linked sugars (Van den Steen *et al.*, 2006). In addition to the secreted MMPs, six human membrane-bound MMPs (MT-MMPs) exist. These are linked to the cell surface through a COOH-terminal transmembrane domain (MT1-, MT2-, MT3- and MT5-MMP) or a glycosyl phosphatidylinositol (GPI) anchor (MT4- and MT6-MMP) (Visse and Nagase, 2003).

Once switched on, MMP proteolytic activity is under tight control by specific inhibitors, primarily the tissue inhibitors of metalloproteinases (TIMPs) (Brew *et al.*, 2000). In plasma, the general protease inhibitor, α_2-macroglobulin, is the predominant MMP inhibitor (Baker *et al.*, 2002), whereas the TIMPs are considered to be the key inhibitors in tissue. In humans, four different TIMPs (TIMP-1 to -4) have been characterized that form non-covalent 1:1 complexes with MMPs. TIMP-1, TIMP-2, and TIMP-4 are present in the extracellular environment in a soluble form, whereas TIMP-3 is insoluble, sequestered by the ECM (Gomez *et al.*, 1997). Although TIMPs bind tightly to most MMPs, some important differences exist in the inhibition profile (Table 1). For instance, TIMP-1 is known to inhibit most soluble MMPs, but it is a poor inhibitor for MT1-MMP, MT2-MMP, MT3-MMP, MT5-MMP, and MMP-19. Binding affinity to MMP-9 is high for TIMP-1, but low for TIMP-2 and TIMP-3, whereas TIMP-2, TIMP-3, and TIMP-4 bind with high affinity to

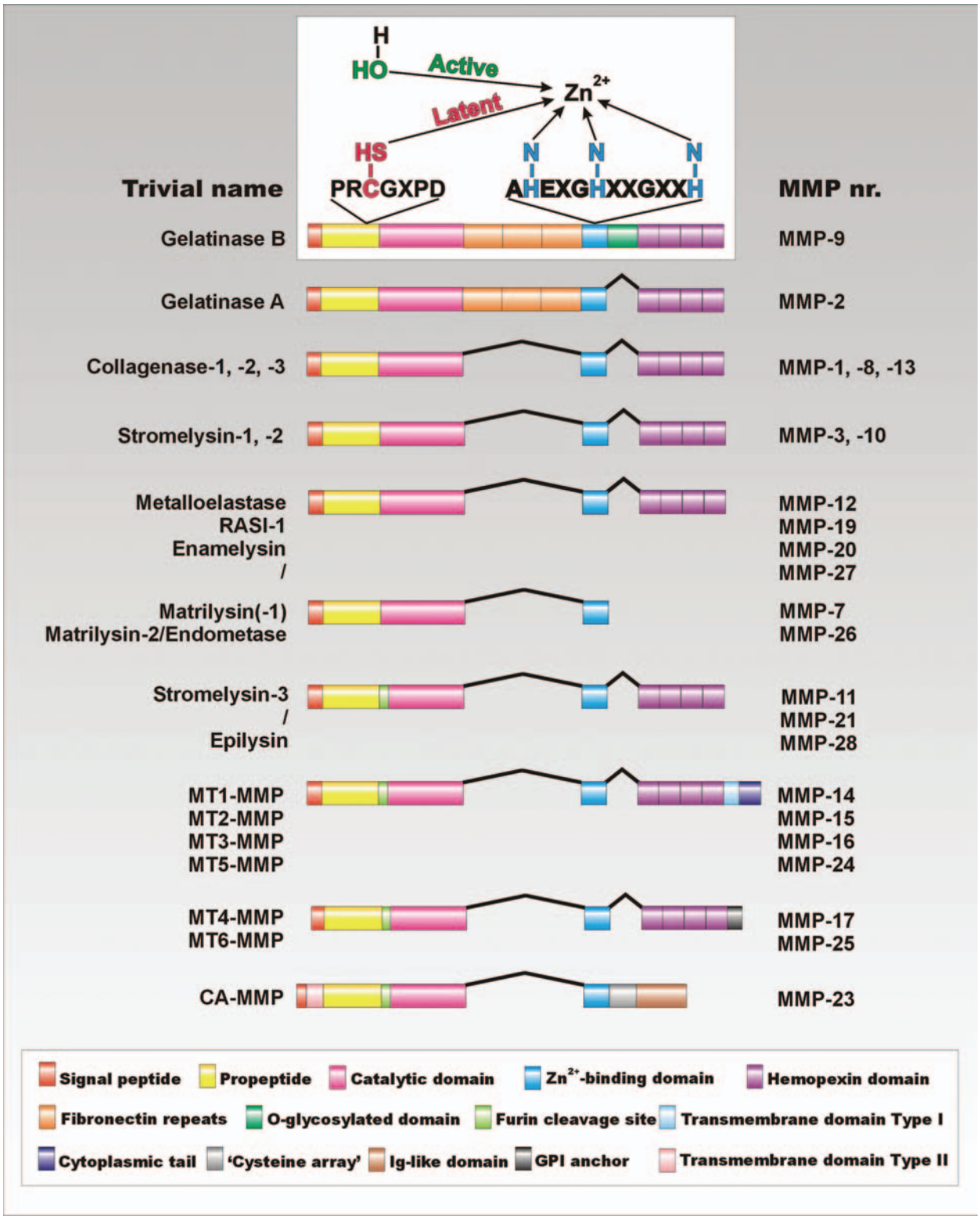

FIGURE 1 Domain structure of the human MMPs. An archetypal MMP contains a signal peptide for secretion, a propeptide, a catalytic domain with a conserved Zn^{2+}-binding motif and a COOH-terminal domain. The hemopexin domain is absent in the smallest MMPs, the matrilysins, whereas the gelatinases incorporate three fibronectin type II repeats for the binding of gelatin, and MMP-9 is the only MMP to possess a Ser/Thr/Pro-rich O-glycosylated domain. Some MMPs are attached to the cell surface through a COOH-terminal transmembrane domain or a GPI anchor. The interaction of a conserved cysteine in the propeptide with the catalytic Zn^{2+} ion seals the catalytic site and results in the latency of the pro-enzyme. MMPs are activated according to the 'cysteine switch mechamism' in which removal of the propeptide frees the catalytic Zn^{2+} ion, allowing it to bind a hydrolytic water ion and the substrate. CA-MMP, cysteine array-MMP; GPI, glycosyl phosphatidylinositol; Ig, Immunoglobulin; RASI-1, rheumatoid arthritis synovial inflammation-1. Based on (Van den Steen *et al.*, 2002), (Folgueras *et al.*, 2004) and (Nagase *et al.*, 2006).

TABLE 1 **TIMP characteristics and inhibition profiles**

	TIMP-1	TIMP-2	TIMP-3	TIMP-4
Localization	Soluble	Soluble/cell surface	ECM	Soluble/cell surface
MW (kDa)	28	21	24/27	22
MMPs inhibited	Most MMPs, best inhibited:MMP-9	Many MMPs, best inhibited: MMP-2	Many MMPs, best inhibited: MMP-2	Many MMPs, best inhibited: MMP-2
MMPs poorly inhibited	MT1-MMP MT2-MMP MT3-MMP MT5-MMP MMP-19	None	None	None
ADAMs inhibited	ADAM-10	None	ADAM-10 ADAM-12 ADAM-17 ADAM-19 ADAMTS-4 ADAMTS-5	none

MMP-2 (Gomez *et al.*, 1997; Nagase *et al.*, 2006). In addition, TIMP-3 inhibits members of the 'a disentegrin and metalloproteinase (ADAM)-family', including ADAM-10/Kuzbanian, ADAM-12, ADAM-17/tumor necrosis factor-α (TNF-α)-converting enzyme (TACE) and ADAM-19, as well as the aggrecan-degrading enzymes 'a disentegrin and metalloproteinase with thrombospondin-like motif' (ADAMTS)-4 and ADAMTS-5, and TIMP-1 inhibits ADAM-10 (Baker *et al.*, 2002). As a consequence, the TIMP inhibition profile of a particular cleavage can already shed some light on the identity of the protease in charge.

Thanks to their structural diversity and broad substrate range, MMPs play a part in multiple physiological and pathological processes (Sternlicht and Werb, 2001). As it is more challenging to study membrane-bound molecules, the initial identification of MMP substrates included mainly soluble proteins. However, due to the fast development and fine-tuning of powerful biochemical techniques, insights into MMP cleavage of membrane-bound substrates grew in parallel with the appreciation of its relative importance in physiology as well as pathology. This review contains a survey of known membrane-bound substrates of every MMP, or more specifically of all proteins with a transmembrane domain that have been shown to be the subject of modification by one or several MMPs. In addition, we will discuss the cleavage of some important cell surface proteins that do not contain a transmembrane domain but are always attached to the cell surface by other means of anchoring. The consequences of proteolysis of cell surface proteins by MMPs will be stressed to generate, from scattered information, a clear view on the importance of this process. Consequently, the substrates are grouped in functional classes in the context of the physiological or pathological roles they play with or without proteolysis. To facilitate comprehensive reading, all substrate molecules are grouped in a master table which includes the modifying MMPs, the context of cleavage, known cleavage sites, the biological effect of proteolysis and the physiopathological implications (Table 2). In many instances, various names, abbreviations and acronyms have been given to these substrates. For clarity, these are provided once at the beginning of each section (see also the list of abbreviations).

1. PROTEOLYTIC MODIFICATION OF CELL SURFACE PROTEINS IN CANCER DEVELOPMENT AND EVOLUTION

Tumorigenesis and cancer progression originate from at least seven fundamental alterations in cellular physiology: 1) production of autocrine growth signals; 2) unsensitivity to growth-inhibitory signals; 3) escape from apoptosis; 4) loss of senescence; 5) sustained angiogenesis; 6) tissue invasion; and 7) metastasis (Hanahan and Weinberg, 2000). MMPs have an impact on the microenvironment of tumors. Moreover, the expression and activity of specific MMPs is elevated in almost every kind of cancer. Some MMPs are synthesized principally by tumor cells (*e.g.*, MMP-7), whereas other MMPs (*e.g.*, MMP-2 and -9) are rather produced by stromal cells in the tumors, like fibroblasts, inflammatory cells, and endothelial cells (Egeblad and Werb,

TABLE 2 **Proteolysis of cell surface proteins by MMPs in physiological and pathological processes**

Substrate[1]	CD[2]	MA[3]	MMP	Cleavage sites	Substrate modulation[4]	Effects on physiology/pathology	Reference
				ADHESION MOLECULES			
pro-α_v-integrin	CD51	TMD	MT1-MMP	Asp891-Leu 892 after Cys852 *In vitro*	Maturation	Activation of $\alpha_v\beta_3$, leading to increased tumor cell adhesion and migration on vitronectin; Cross-talk between $\alpha_v\beta_3$ and $\alpha_2\beta_1$ integrins, leading to increased tumor cell adhesion to type I collagen	(Ratnikov *et al.*, 2002; Deryugina *et al.*, 2002; Baciu *et al.*, 2003)
pro-α_3-integrin	CD49c	TMD	MT1-MMP	*In vitro*	Maturation	ND	(Baciu *et al.*, 2003)
pro-α_5-integrin	CD49e	TMD	MT1-MMP	*In vitro*	Maturation	No influence on $\alpha_5\beta_1$ integrin function	(Baciu *et al.*, 2003)
pro-β_3-integrin	CD61	TMD	MT1-MMP	*In vitro*	Maturation	Activation of $\alpha_v\beta_3$, leading to increased tumor cell adhesion to vitronectin	(Deryugina *et al.*, 2000)
pro-β_4-integrin	CD104	TMD	MMP-7	*In vitro*	Inactivation	Reduced binding of $\alpha_6\beta_4$ to laminin, reducing tumor cell adhesion and migration	(von Bredow *et al.*, 1997; Abdel-Ghany *et al.*, 2001)
tTG	/	CSL	MT1-MMP MMP-2	Pro375-Val376 Arg458-Ala459 His461-Leu462 *In vitro*	Degradation	Suppression of tumor cell adhesion and migration on fibronectin; Stimulation of tumor cell migration on collagen matrices	(Belkin *et al.*, 2001; Belkin *et al.*, 2004)
			MT2,3-MMP	*In vitro*	Degradation	ND	(Belkin *et al.*, 2001; Belkin *et al.*, 2004)
CD44	CD44	TMD	MT1-MMP	Gly192-Tyr193 Gly233-Ser234 (Ser249-Gln250) *In vitro + in vivo*	Inactivation	Stimulation of tumor cell migration and invasion	(Kajita *et al.*, 2001; Nakamura *et al.*, 2004)
			MT2,3,5-MMP	Gly233-Ser234 *In vitro*	Inactivation	ND (Possible stimulation of tumor cell migration and invasion)	(Suenaga *et al.*, 2005)
ICAM-1	CD54	TMD	MMP-9	Between Arg441 and the TMD	Inactivation	Protection of tumor cells against their elimination by cytotoxic T cells and NK cells	(Fiore *et al.*, 2002; Sultan *et al.*, 2004)
L-selectin	CD62L	TMD	MMP-1 MMP-3	*In vitro*	ND	ND (Possible effect on leukocyte rolling, transendothelial migration, activation and T-cell reentry into the peripheral lymph nodes after activation)	(Preece *et al.*, 1996)
				APOPTOSIS MEDIATORS			
FasL	CD178	TMD	MMP-7	Glu110-Leu111 Glu113-Leu114 Ser126-Leu127 Glu142-Leu143 *In vitro + in vivo*	sEffector* with lower activity**	Apoptosis induction in epithelial cells* but reduced apoptosis induction in tumor cells**	(Tanaka *et al.*, 1998; Powell *et al.*, 1999; Mitsiades *et al.*, 2001; Vargo-Gogola *et al.*, 2002a)

				In vitro		Protection of neuron cultures from Aβ-induced neuronal cell apoptosis**, possibly preventing neuronal loss in Alzheimer's disease	(Ethell *et al.*, 2002)
			MMP-3	Glu110-Leu111 Glu113-Leu114 Ser126-Leu127 *In vitro*		Reduced intra-articular apoptosis** leading to synovial proliferation in rheumatoid arthritis	(Matsuno *et al.*, 2001)
Fas	CD95	TMD	MMP-7	Glu19-Leu20 Asn32-Leu33 *In vitro*	Down-regulation	Reduced oligomerization of Fas receptors leading to reduced sensitivity of tumor cells to Fas-mediated apoptosis	(Strand *et al.*, 2004)
Death receptor-6	/	TMD	MT1-MMP	*In vitro*	ND	ND	(Overall *et al.*, 2004)
					RECEPTORS		
FGFR-1	CD331	TMD	MMP-2	Val368-Met369 *In vitro*	sInhibitor	Inhibition of FGF-mediated stimulation of tumor cell growth	(Levi *et al.*, 1996)
HER2	CD340	TMD	ND MMP	*In vitro*	Potentiation	Enhanced oncogenic potential including promotion of excessive and autonomous tumor cell growth, increased tumor cell survival, neovascularisation and invasiveness	(Codony-Servat *et al.*, 1999)
uPAR	CD87	GPI	MMP-12	Thr86-Tyr87 *In vitro* + *ex vivo*	Inactivation + Activation	- Destruction of the binding site for u-PA and vitronectin, decreasing cell-surface associated u-PA activity (= inactivation) and reducing tumor-induced angiogenesis + generation of uPAR molecules with chemotactic activities (= activation) - Destruction of u-PA-dependent invasion, proliferation and morphogenesis of endothelial cells, leading to vascular alterations in systemic sclerosis patients	(Koolwijk *et al.*, 2001; Andolfo *et al.*, 2002; D'Alessio *et al.*, 2004; Serrati *et al.*, 2006)
			MMP-3	Thr86-Tyr87 *In vitro*	Inactivation + Activation	Destruction of the binding site for u-PA and vitronectin, decreasing cell-surface associated u-PA activity (= inactivation) and reducing tumor-induced angiogenesis + generation of cell-surface activated uPAR molecules with chemotactic activities (= activation)	(Andolfo *et al.*, 2002)
			MT6-MMP	Thr86-Tyr87 Tyr87-Ser88 Ser88-Arg89 Arg89-Ser90 *In vitro*			

(Continued on next page)

TABLE 2 **Proteolysis of cell surface proteins by MMPs in physiological and pathological processes *(Continued)***

Substrate[1]	CD[2]	MA[3]	MMP	Cleavage sites	Substrate modulation[4]	Effects on physiology/pathology	Reference
			MMP-19	Thr86-Tyr87 Tyr87-Ser88 Arg89-Ser90 *In vitro*			
			MMP-2,-9 MMP-8,-13 MT1-MMP	*In vitro*	ND	ND	(Andolfo *et al.*, 2002)
Semaphorin 4D	CD100	TMD	MT1-MMP	*In vitro* + *in vivo*	sEffector	Promotion of tumor-induced angiogenesis	(Basile *et al.*, 2007)
LR	/	TMD	*X*MMP-11	Ala115-Phe116 Pro133-Ile134 *In vitro*	ND (sEffector)	ND (Possible enhancement of tumor cell invasion and angiogenesis)	(Amano *et al.*, 2005b)
*X*LR	/	TMD	*X*MMP-11	Ala115-Phe116 Pro133-Ile134 *In vitro* + *in vivo*	ND (sEffector)	ND (Possible promotion of larval epithelial cell apoptosis needed for intestinal metamorphosis in *Xenopus laevis*)	(Amano *et al.*, 2005a, 2005b)
			MMP-2,-3–9 MT1-MMP	*In vitro*	ND	ND	(Amano *et al.*, 2005b)
EMMPRIN	CD147	TMD	MT1-MMP MT2-MMP	Asn98-Ile99 Pro93-Met94 *In vitro*	Inactivation + sEffector	Downregulation of cellular EMPRINN functions, possible diffusion and amplification of EMPRINN activities on adjacent or more distal cells, promoting tumor growth, metastasis, angiogenesis and multidrug resistance	(Egawa *et al.*, 2006)
			MMP-1 MMP-2	*In vitro*	Inactivation + sEffector	Amplification cascade of MMP activity, leading to enhanced ECM degradation in atherosclerotic plaques, promoting plaque growth and plaque destabilization	(Haug *et al.*, 2004)
LRP	CD91	TMD	MT1,2,3,4-MMP	*In vitro*	Inactivation	Suppression of clearance of ECM-degrading proteases, promoting tissue remodelling by migrating tumor cells	(Rozanov *et al.*, 2004a)
PAR1	/	TMD	MMP-1	Arg41-Ser42 *In vitro* + *in vivo*	Activation	Promotion of cancer cell migration and invasion + induction of endothelial cell activation	(Boire *et al.*, 2005; Pei, 2005; Goerge *et al.*, 2006)
IL-2Rα	CD25	TMD	MMP-9	*In vitro*	Inactivation	Reduced proliferation of tumor-infiltrating cytotoxic T cells, leading to tumor immune escape	(Sheu *et al.*, 2001)
CD14	CD14	GPI	MMP-12	*In vitro* + *in vivo*	Inactivation + sEffector	Reduced innate host defence activities such as impairment of LPS uptake and reduced LPS-induced TNF-α production by alveolar macrophages	(Senft *et al.*, 2005)
			MMP-1	*In vitro*	ND	ND	(Bryniarski *et al.*, 2003)
					CHEMOKINES		
Fractalkine	/	TMD	MMP-2	*In vitro*	sEffector + sInhibitor	ND	(Overall and Dean, 2006)

					CYTOKINES		
RANKL	CD254	TMD	MMP-7	Met145-Met146 *In vitro + in vivo*	sEffector	Promotion of prostate cancer-induced osteolysis	(Lynch *et al.*, 2005)
			MT1-MMP			Downregulation of local osteoclastogenesis and bone resorption	(Hikita *et al.*, 2006)
			MMP-3	*In vitro*	ND	ND	(Lynch *et al.*, 2005)
			MT2,3,5-MMP	Met145-Met146 *In vitro*	ND	ND	(Hikita *et al.*, 2006)
pro-TNF-α	/	TMD	MMP-1	Ala74-Gln75 Ala76-Val77 *In vitro*	sEffector	Induction of inflammation in response to bacteria and pathogen-associated molecular patterns; Excess TNF-α in Crohn's disease, multiple sclerosis, rheumatoid arthritis and septic shock	(Gearing *et al.*, 1994; Gearing *et al.*, 1995; Chandler *et al.*, 1996; d'Ortho *et al.*, 1997; English *et al.*, 2000; Mohan *et al.*, 2002)
			MMP-2,-3	*In vitro*			
			MMP-9	Ala74-Gln75			
			MT4-MMP	*In vitro*			
			MT1-MMP	Ala76-Val77			
			MT2-MMP	before Leu113 *In vitro*			
			MMP-7	Ala76-Val77 *In vitro + ex vivo*		Induction of MMP-3, leading to spontaneous resorption of herniated discs	(Haro *et al.*, 2000a; Haro *et al.*, 2000b; Mohan *et al.*, 2002)
			MMP-12	*In vitro + in vivo*		Induction of acute cigarette smoke-provoked inflammation	(Churg *et al.*, 2003)
pro-IL-1β	/	CSL	MMP-2,-3,-9	*In vitro*	Activation	Induction of inflammation; Stimulation of tumor invasion and angiogenesis	(Schönbeck *et al.*, 1998)
IL-1β	/	S	MMP-1,-3,-9	*In vitro*	Degradation	Downregulation of the pro-inflammatory, metastatic and angiogenic effects of active IL-1β	(Ito *et al.*, 1996)
			MMP-2	Glu25-Leu26 *In vitro*			
KitL	/	TMD	MMP-9	*In vivo*	sEffector	Maintenance of leukocyte homeostasis in the blood; Stimulation of proliferation of quiescent smooth muscle cells, leading to intimal hyperplasia	(Heissig *et al.*, 2002; Hollenbeck *et al.*, 2004)

(Continued on next page)

TABLE 2 **Proteolysis of cell surface proteins by MMPs in physiological and pathological processes *(Continued)***

Substrate[1]	CD[2]	MA[3]	MMP	Cleavage sites	Substrate modulation[4]	Effects on physiology/pathology	Reference
pro-TGF-β	/	CSL	MMP-2,-3,-9 MT1-MMP	*In vitro*	Activation	Tumor suppression in early stages of tumorigenesis, but stimulation of angiogenesis and metastasis in later stages of cancer development	(Yu and Stamenkovic, 2000)
					GROWTH FACTORS		
HB-EGF	/	TMD	MMP-7	*In vitro* + *in vivo*	sEffector	- Regulation of postpartum uterine and mammary gland involution and maintenance of lactation - Pathological vasoconstriction and growth of smooth muscle cells; Induction of tumor cell growth and angiogenesis	(Iwamoto and Mekada, 2000; Roelle *et al.*, 2003; Razandi *et al.*, 2003; Milenkovic *et al.*, 2003; Hao *et al.*, 2004; Lucchesi *et al.*, 2004; Ongusaha *et al.*, 2004)
			MMP-2	*In vitro* + *in vivo*		- Signal transduction induced by GnRH and estradiol	
			MMP-9	*In vitro* + *in vivo*		- Signal transduction induced by GnRH and estradiol; Proliferation of glia cells in PVR	
			MMP-3	Glu151-Asn152 *In vitro*		- Induction of tumor cell growth and angiogenesis	(Iwamoto and Mekada, 2000; Ongusaha *et al.*, 2004)
					PROTEASES		
MT1-MMP	/	TMD	MT1-MMP	Gly284-Gly285 Ala255-Ile256 *In vitro*	Inactivation + mInhibitor	Downregulation of MT1-MMP-mediated promotion of tumor invasion and metastasis	(Toth *et al.*, 2002)
			MT3-MMP	*In vitro*	Inactivation	Downregulation of MT1-MMP activity in smooth muscle cells, gliomas or injured blood vessels	(Shofuda *et al.*, 2001)
ADAMTS-4	/	CSL	MT4-MMP	Lys694-Phe685 Thr581-Phe582 *In vitro*	sEffector	Degradation of aggrecan in rheumatoid arthritis	(Gao *et al.*, 2002; Gao *et al.*, 2004)
			MMP-9,-13	*In vitro*	Activation	ND	(Tortorella *et al.*, 2005)

INTERCELLULAR JUNCTION PROTEINS							
E-cadherin	CD324	TMD	MMP-7	*In vitro* + *in vivo*	sInhibitor	Induction of tumor cell invasion and metastasis; rounding of apoptotic cells and tumor cells, promoting tumor cell exit from the epithelium; repair of injured lung epithelium	(Steinhusen *et al.*, 2001; Davies *et al.*, 2001; Noe *et al.*, 2001; McGuire *et al.*, 2003)
			MMP-3	*In vitro*	sInhibitor	Induction of tumor cell invasion and metastasis; promotion of epithelial-mesenchymal transition; rounding of apoptotic cells and tumor cells, promoting tumor cell exit from the epithelium	(Lochter *et al.*, 1997; Steinhusen *et al.*, 2001; Noe *et al.*, 2001)
			MT1-MMP	*In vitro*	Degradation	Disruption of cell-cell attachments during renal ischemia, possibly causing intra-tubular obstructions in acute renal failure	(Covington *et al.*, 2006)
N-cadherin	CD325	TMD	MT1-MMP	*In vitro*	Degradation	Disruption of cell-cell attachments during renal ischemia, possibly causing intra-tubular obstructions in acute renal failure	(Covington *et al.*, 2006)
			MT5-MMP	*In vitro*	ND	ND (Possible role in mechanisms of synaptic regulation)	(Monea *et al.*, 2006)
VE-cadherin	CD144	TMD	MMP-7	*In vitro*	ND	Acceleration of endothelial cell proliferation	(Ichikawa *et al.*, 2006)
Occludin	/	IM	MMP-2	*In vitro*	Degradation	BRB disruption leading to retinal edema in PDR	(Giebel *et al.*, 2005)
			MMP-9	*In vitro* + *in vivo*	Degradation	BRB disruption leading to retinal edema in PDR + disruption of corneal epithelial barrier function, causing ocular irritation and visual morbidity in KS Disruption of endothelial tight junctions during renal ischemia and possible degradation of the vascular basement membrane, leading to acute renal failure	(Giebel *et al.*, 2005; Pflugfelder *et al.*, 2005; Caron *et al.*, 2005)
			MMP-3	*In vitro* + *in vivo*	Degradation	BBB disruption in neuroinflammation	(Gurney *et al.*, 2006)
			MMP-7	*In vitro*	Degradation	Estrogen-mediated increase of paracellular permeability in vaginal-cervical epithelia	(Gorodeski, 2007)
Claudin-5	/	IM	MMP-3	*In vitro* + *in vivo*	Degradation	BBB disruption in neuroinflammation	(Gurney *et al.*, 2006)

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TABLE 2 **Proteolysis of cell surface proteins by MMPs in physiological and pathological processes *(Continued)***

Substrate[1]	CD[2]	MA[3]	MMP	Cleavage sites	Substrate modulation[4]	Effects on physiology/pathology	Reference
				STRUCTURAL PROTEINS			
Betaglycan	/	TMD	MT1-MMP MT3-MMP	*In vitro*	sInhibitor	Inhibition of TGF-β-induced tumor angiogenesis	(Velasco-Loyden *et al.*, 2004; Bandyopadhyay *et al.*, 2005)
MUC1	CD227	TMD	MT1-MMP	*In vitro*	ND	Possible role in the defense of epithelial surfaces like uterine, lung and intestinal epithelia; Possible influence on tumor cell proliferation, metastasis and immune evasion	(Thathiah and Carson, 2004)
Syndecan-1	CD138	TMD	MT1,3-MMP	Gly245-Leu246 *In vitro*	Inactivation	Enhanced tumor cell migration on collagen	(Endo *et al.*, 2003)
			MMP-7	*In vitro* + *in vivo*	sEffector	Release of a syndecan-1/KC complex from the mucosal surface of injured lungs, forming a chemokine gradient that directs neutrophils to the site of injury	(Li *et al.*, 2002)
			MMP-9	*In vitro*	Inactivation	ND	(Brule *et al.*, 2006)
Syndecan-3	/	TMD	ND MMP	*In vitro* + *In vivo*	Inactivation	Abrogation of Schwann cell adhesion to α4(V) collagen *in vitro* and in the peripheral nerve tissue of newborn rats during the myelin-forming process in Schwann cells	(Asundi *et al.*, 2003)
Syndecan-4	/	TMD	MMP-9	*In vitro*	Inactivation	ND	(Brule *et al.*, 2006)
BP180	/	TMD	MMP-9	*In vitro*	Degradation	Disruption of BP180-mediated anchoring of dermis and epidermis (*in vitro*), possibly leading to blister formation in BP	(Liu *et al.*, 1998)
MBP	/	IM	MMP-9	Phe90-Lys91 Ser110-Leu111 Phe114-Ser115 Asp133-Tyr134 *In vitro* + *in vivo*	Degradation	Demyelination and generation of encephalitogenic peptides, causing neuroinflammation in multiple sclerosis	(Proost *et al.*, 1993; Chandler *et al.*, 1995; Asahi *et al.*, 2001)
			MMP-1,-2,-3 MMP-7,-12	*In vitro*	Degradation	ND	(Chandler *et al.*, 1995; Chandler *et al.*, 1996)
NG2 proteoglycan	/	TMD	MMP-9	*In vitro* + *in vivo*	Degradation	Removal of NG2 proteoglycan-mediated inhibition of oligodendrocyte maturation and differentiation, thus promoting remyelination after CNS injury	(Larsen *et al.*, 2003)

β dystroglycan	/	TMD	MMP-2,-9	*In vitro + in vivo*	Degradation	BBB breakdown, CNS infiltration by leukocytes and development of multiple sclerosis disease symptoms	(Agrawal *et al.*, 2006)
APP	/	TMD	MMP-2	Lys687-Leu688* Glu668-Val669** *In vitro*	sEffector	Release of sAPPα*, preventing Aβ formation in Alzheimer's disease; or release of sAPPβ**, promoting Aβ formation; inhibition of MMP-2 by sAPP	(Miyazaki *et al.*, 1993, 1994; LePage *et al.*, 1995)
			MMP-3	Glu668-Val669 Glu674-Phe675 *In vitro*	ND	ND	(Rapala-Kozik *et al.*, 1998)
			MT1-MMP	Asn579-Met580# *In vitro*	sEffector	Release of sAPPtrc#, which displaces the MMP-2 inhibitory activity of APP or sAPP, promoting MMP-2-catalyzed ECM degradation and tumor cell migration; or release of sAPPα##, reducing Aβ formation in Alzheimer's disease	(Higashi and Miyazaki, 2003b)
			MT3-MMP	Ala463-Met464 Asn579-Met580# His622-Ser623 His685-Gln686## *In vitro*	sEffector		(Ahmad *et al.*, 2006)
			MT5-MMP	ND *In vitro*	ND	ND	(Ahmad *et al.*, 2006)
Soluble Aβ	/	S	MMP-3	*In vitro*	Degradation	Possible reduction of the accumulation of extracellular Aβ peptides in toxic amyloid plaques during Alzheimer's disease	(White *et al.*, 2006)
			MMP-2	Lys16-Leu17 Leu34-Met35 Met35-Val36 *In vitro + in vivo*			(Roher *et al.*, 1994; White *et al.*, 2006; Yin *et al.*, 2006)
			MMP-9	Lys16-Leu17 Phe20-Ala21 Asp23-Val24 Ala30-Ile31 Gly33-Leu34 Leu34-Met35 Gly37-Gly38 *In vitro + in vivo*			(Backstrom *et al.*, 1996; Yan *et al.*, 2006; Yin *et al.*, 2006)
Aβ fibrils	/	S	MMP-2,-9	Phe20-Ala21 Ala30-Ile31 *In vitro*	Degradation	Degradation of Aβ fibrils in amyloid plaques, possibly contributing to the clearance of plaques from Alzheimer's diseased brains	(Yan *et al.*, 2006)

Aβ, β-amyloid protein;APP, amyloid precursor protein; BBB, blood-brain barrier; BP, bullous pemphigoid; BRB, blood-retinal barrier; CNS, central nervous system; GnRH, gonadotropin-releasing hormone; KS, keratitis sicca; PDR, proliferative diabetic retinopathy; PVR, proliferative vitreoretinopathy; *X*, *Xenopus*.

[1]Substrate: for the meaning of the acronyms, see the list of abbreviations.

[2]CD: Cluster of differentiation of human (glyco)proteins.

[3]MA: type of cell membrane association: TMD, transmembrane domain; CSL, cell surface localization; GPI, glycosyl phosphatidylinositol anchor; S, soluble; IM, integral membrane protein.

[4]Substrate modulation: mInhibitor, membrane-bound inhibitor; sInhibitor, release of soluble inhibitor; sEffector, release of soluble effector; ND, not defined.

2002). In earlier studies, the MMPs were claimed to be important in migration, invasion and metastasis mainly by the degradation of basement membranes and structural components in the ECM. However, more recent findings show that proteolysis of a panacea of essential secreted and membrane-bound molecules, like growth factor precursors and receptors, tyrosine kinase receptors (TKRs), cytokines and chemokines, cell adhesion molecules, and other proteases, modifies the tumor microenvironment. These modifications will affect tumor progression at all levels (Nelson *et al.*, 2000; McCawley and Matrisian, 2001). In this first part we will discuss proteolytic modifications of cell surface proteins by MMPs and their concomitant effects on cancer evolution (see Figure 2). Insight into the paradoxical consequences of proteolysis of cell surface proteins by MMPs might be of paramount importance in the design of new cancer therapies based on MMP inhibition.

1.1 Promotion or Inhibition of Cancer Cell Proliferation

MMPs stimulate tumor cell proliferation by catalysing the release of growth factors from the ECM, by the activation of membrane-bound growth factor receptors or by cleavage of membrane-bound substrates, e.g., integrins, that assist in the induction of cell proliferation. Conversely, MMPs might also negatively regulate cancer-cell growth by releasing pro-apoptotic molecules like Fas ligand (FasL) and TNF-α, or by activating transforming growth factor-β (TGF-β), which has a tumor suppressing effect in early phases of oncogenesis (Egeblad and Werb, 2002).

1.1.1 Fibroblast Growth Factor Receptor-1 (FGFR-1)

The fibroblast growth factors (FGFs) constitute a family of twenty structurally related polypeptides with a common high affinity to heparin. Their biological functions are broad and range from the induction of cellular proliferation to tissue regeneration, neurite outgrowth, and angiogenesis (Powers *et al.*, 2000). FGFs elicit their biological response by binding to four different cell surface TKRs, which are typically composed of an extracellular part with three immunoglobulin (Ig)-like domains, a single transmembrane domain and a bipartite TKR domain. Regulation of FGF biological activity may be achieved through several mechanisms, including binding to high and low affinity receptors on the cell surface, release of FGF from the ECM by heparanases or other proteases or binding to a carrier protein that can deliver FGFs to their receptors. In addition, FGF activity might be regulated by the release of the entire ectodomain of the FGF receptor-1 (FGFR-1, basic fibroblast growth factor receptor-1 (bFGF-R), fms-like tyrosine kinase 2, c-fgr, CD331) into the circulation and the ECM (Levi *et al.*, 1996; Powers *et al.*, 2000). MMP-2 is able to free the entire FGFR-1 ectodomain from an immobilized FGFR1 ectodomain-alkaline phosphatase (FRAP) fusion protein *in vitro* by hydrolyzing the Val368-Met369 bond, eight amino acids upstream of the transmembrane domain (Figure 3). By this truncation, the ligand binding site is released as a soluble ectodomain retaining its FGF binding ability (Levi *et al.*, 1996). FGFs may stimulate tumor cell growth in at least one of the following ways: 1) by acting as mitogens for the tumor cells themselves; 2) by promoting angiogenesis for the growing tumor and 3) by inhibiting apoptosis and allowing tumor cells to keep growing beyond normal constraints (Powers *et al.*, 2000). In this regard, release of the FGFR-1 ectodomain by MMP-2 would counter these mechanisms in two ways. On the one hand, the released FGFR-1 ectodomain might bind extracellular FGF and in this manner diminish the biological availability and growth promoting activity of FGF. On the other hand, treatment of FGFR-1 overexpressing cells with MMP-2 clearly reduces binding of the FGF mitogen (Levi *et al.*, 1996). Thus, investigating the release of the FGFR-1 ectodomain by MMP-2 or by related MMPs *in vivo* may provide new insights in tumor cell growth inhibition.

1.1.2 Heparin-Binding Epidermal Growth Factor-Like Growth Factor (HB-EGF)

The heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF, also called diphtheria toxin receptor [DT-R]) is a member of the EGF family of growth factors, which encompasses the receptor family of a number of structurally homologous mitogens like EGF, transforming growth factor-α (TGF-α) and amphiregulin (AR) (Raab and Klagsbrun, 1997; Iwamoto and Mekada, 2000). HB-EGF binds to the EGF receptor (EGFR/HER1/ErbB1) as well as to HER4/ErbB4, inducing homo- or heterodimerisation of the monomeric receptors with consequent tyrosine phosphorylation in the cytoplasmic domains, resulting in the activation of several signal tranduction pathways (Zwick *et al.*, 1999; Herbst, 2004). HB-EGF is synthesized as a transmembrane protein (mHB-EGF) with the ectodomain

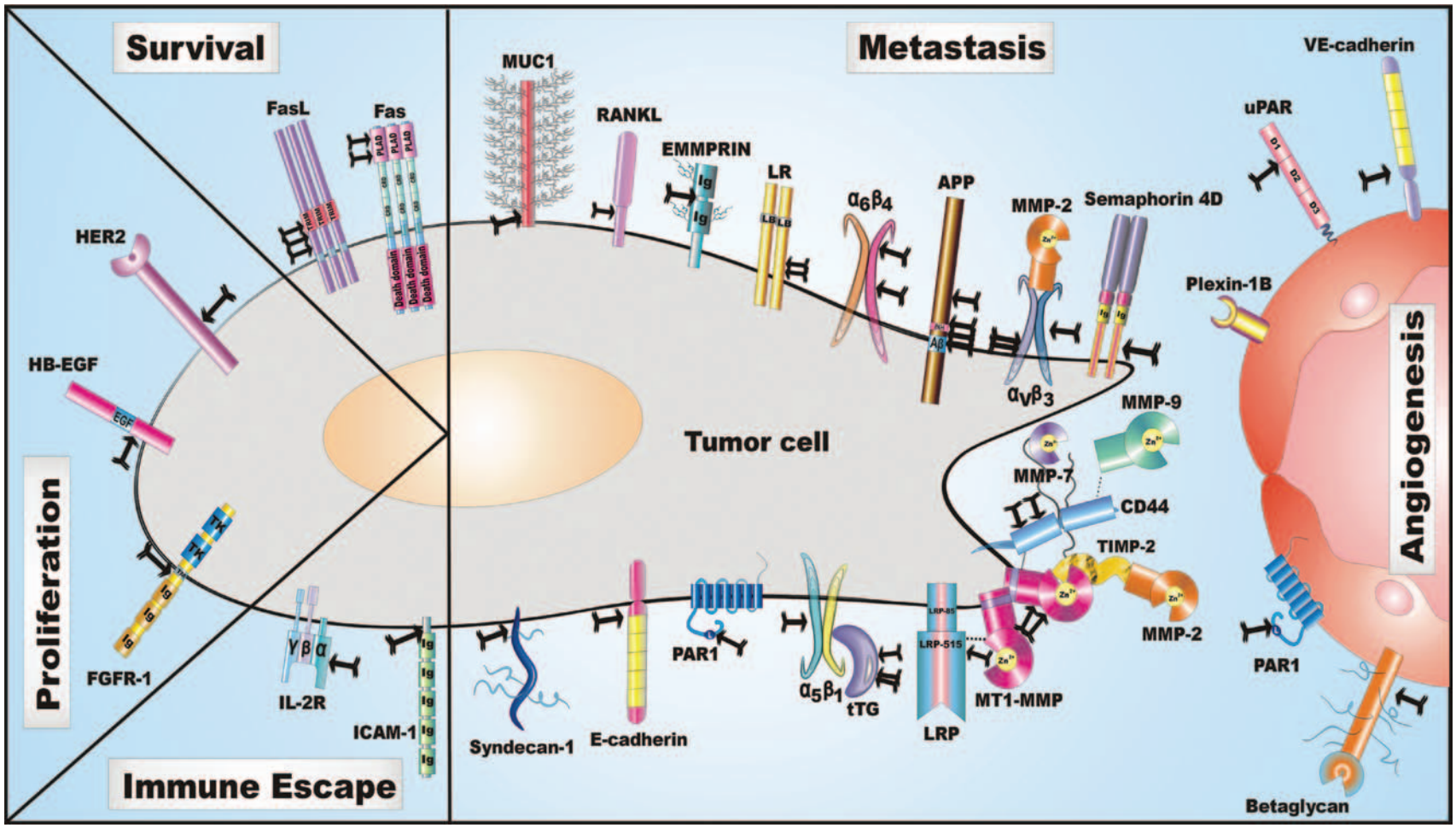

FIGURE 2 Proteolytic modification of cell surface proteins by MMPs in cancer development and evolution. All cell surface-associated molecules cleaved by MMPs during cancer development are presented on the cell membrane of a migrating tumor cell. These susbstrates are grouped according to the most affected cancer mechanism. Ectodomain cleavage of growth factor receptors may amplify the promotion of cell proliferation (HER2) or on the contrary release a soluble decoy receptor that binds soluble growth factors, thus decreasing their binding to intact growth-promoting receptors (FGFR-1). Proteolysis of FasL and Fas disturbs trimerization, which attenuates apoptosis induction, allowing the tumor cells to survive and proliferate. Formation of new vessels is crucial for the survival of a growing tumor. Cleavage of proteins on the endothelial cell membranes by MMPs may have a pro-angiogenic (VE-cadherin, PAR1) as well as an anti-angiogenic effect (uPAR, Betaglycan). Furthermore, alternation of adhesion and anti-adhesion is required as the cell migrates during the processes of invasion and metastasis. MMPs promote metastasis by proteolysis of adhesion molecules (integrin precursors, tTG, CD44, E-cadherin), cytokines (RANKL), receptors (PAR1, EMMPRIN, LRP) and structural proteins (syndecan-1, APP). Many of these cleavages are mediated by MT1-MMP, which colocalizes with CD44 at the ruffling edge of migrating tumor cells. CD44 forms a platform to cluster MMPs, which stimulate migration by cleaving substrates on the cell surface and by degrading the ECM. Finally, cleavage of the transmembrane proteins, IL-2Rα and ICAM-1 on transformed leukocytes, allows tumor cells to escape assaults from the immune system. Arrows indicate cleavages by MMPs. For details of the substrate acronyms, see the list of abbreviations.

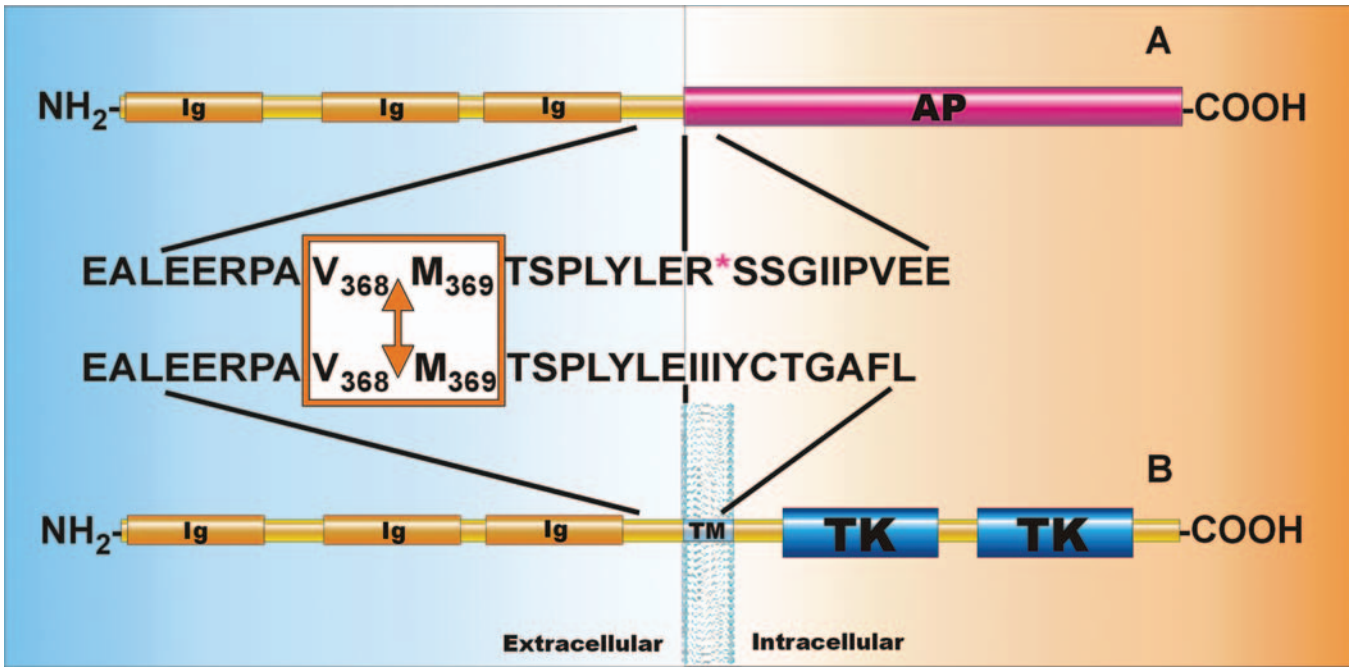

FIGURE 3 **Cleavage of the human FGFR-1 ectodomain-AP fusion protein by MMP-2 and comparison with murine FGFR-1. MMP-2 cleaves the FGFR-1 ectodomain from an immobilized FGFR1 ectodomain-alkaline phosphatase (FRAP) fusion protein *in vitro* at the Val368-Met369 bond, eight amino acids upstream of the transmembrane domain (A). This truncation releases a soluble ectodomain that retains FGF binding ability, thus decreasing FGF binding sites on the tumor cell and competing with the remaining intact FGFR-1. Murine FGFR-1 is shown for comparison (B). Arrow, cleavage site of MMP-2; AP, alkaline phosphatase; Ig, immunoglobulin domain; TK, tyrosine kinase domain; TM, transmembrane domain; R*, start of the AP. Adapted from (Levi *et al.*, 1996).**

containing a heparin-binding and an EGF-like domain. mHB-EGF can be cleaved at the plasma membrane to yield soluble HB-EGF (sHB-EGF) (Raab and Klagsbrun, 1997; Iwamoto and Mekada, 2000). mHB-EGF is not only a precursor for sHB-EGF but is a bioactive molecule itself, which inhibits growth of neighboring cells. As a contrast, sHB-EGF is a potent mitogen for a number of cells including smooth muscle cells, epithelial cells and keratinocytes. Therefore, release of HB-EGF causes the conversion of a juxtacrine growth inhibitor into a paracrine/autocrine growth factor, with an opposite impact on cell growth (Iwamoto and Mekada, 2000; Higashiyama, 2004). mHB-EGF proteolysis is probably mediated by metalloproteinases as it is prevented by the broad spectrum metalloproteinase inhibitor batimastat (Prenzel *et al.*, 1999). In cell cultures, MMP-3 cleaves mHB-EGF in the juxtamembrane domain at the Glu151-Asn152 site (Suzuki *et al.*, 1997; Wu *et al.*, 2004), whereas MMP-7, MMP-2 and MMP-9 and some proteases of the ADAM family [ADAM-9, ADAM-10, ADAM-12, and ADAM-17/TACE] mediate mHB-EGF cleavage *in vivo* under specific circumstances (Higashiyama, 2004; Hao *et al.*, 2004).

HB-EGF plays an important role in a multitude of biological processes and diseases. According to the concerned process, the mHB-EGF proteolysis is induced by varying stimuli and mediated by different metalloproteinases. Release of sHB-EGF allows cross-talking between G protein-coupled receptors (GPCRs) and TKRs in accordance with the triple membrane-passing signal mechanism (TMPS) for the transactivation of TKRs (Figure 4). In the TMPS, GPCRs are activated by an extracellular ligand and subsequently induce the activation of metalloproteinases through the initiation of several signaling cascades. Finally, metalloproteinase activity releases sHB-EGF that activates its TKR, the EGFR (Higashiyama, 2004; Shah and Catt, 2004a). Several studies showed enhanced HB-EGF gene expression in tumors compared to normal tissue (Raab and Klagsbrun, 1997). sHB-EGF expression increased tumor growth rate, colony-forming ability, and activation of the cyclin D1 promotor, as well as induction of vascular endothelial growth factor (VEGF) *in vitro*. In addition, sHB-EGF induced the expression and activity of MMP-3 and MMP-9, leading to enhanced cell migration. *In vivo* sHB-EGF enhanced tumorigenesis and angiogenesis. Hence, release of sHB-EGF by MMPs may be an interesting step for therapeutical intervention (Ongusaha *et al.*, 2004; Miyamoto *et al.*, 2004).

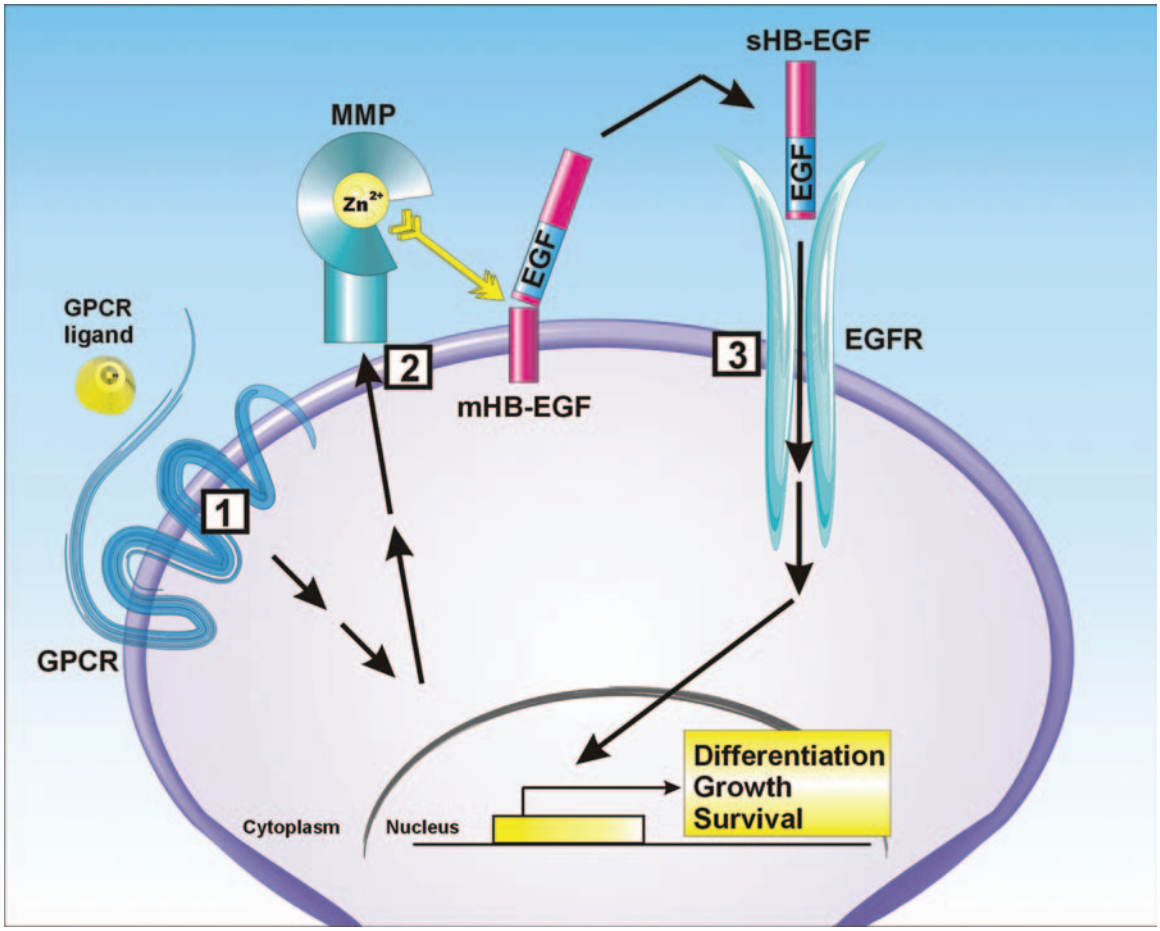

FIGURE 4 **Release of sHB-EGF by MMPs allows cross-talking between G protein-coupled receptors (GPCRs) and tyrosine kinase receptors (TKRs) in accordance with the triple membrane-passing signal mechanism (TMPS). In the TMPS, GPCRs are first activated by an extracellular ligand (1) and subsequently induce the production of metalloproteinases through the initiation of several signaling cascades (2). Finally, metalloproteinase activity releases sHB-EGF that activates its TKR, the EGFR, which induces signal transduction to stimulate cell differentiation, growth and survival (3). Arrow, cleavage by an MMP. Adapted from (Shah and Catt, 2004a).**

Besides stimulating tumor cell proliferation, sHB-EGF induces proliferation of Müller glial cells, which is a common feature of several diseases of the sensory retina. During proliferative vitreoretinopathy (PVR), Müller cells proliferate continuously, migrate onto retinal surfaces, and participate in the formation of periretinal cellular membranes (Rentsch, 1973; Bringmann and Reichenbach, 2001). Proliferation of Müller cells in PVR seems to be stimulated by binding of ATP to P2Y purine receptors (GPCRs) and requires the transactivation of two TKRs. Activation of P2Y receptors by ATP leads to an increase in intracellular Ca^{2+} concentration and may cause a release of platelet-derived growth factor (PDGF) from the cells. Released PDGF activates the PDGF-α receptor (1st TKR), which causes MMP-9 production. MMP-9 then releases sHB-EGF, which in turn activates the EGFR (2nd TKR). Finally, the activated EGFRs and PDGF-α receptors induce proliferative activity in Müller cells by initiating several kinase signaling pathways. As a neutralizing antibody against MMP-9 reversed the mitogenic effect of ATP, inhibition of MMP-9 in the pathway may be a useful tool to suppress uncontrolled intraocular proliferation in PVR (Milenkovic *et al.*, 2003; Shah and Catt, 2004a).

Besides the stimulating effects on tumor progression and PVR, proteolysis of mHB-EGF by MMPs plays a part in cardiovascular diseases (see Section 2.1.1) and reproductive endocrinology (see Section 5.2), which will be discussed in later chapters.

1.1.3 Tyrosine Kinase-Type Cell Surface Receptor HER2

The EGFR family comprises four TKRs of which tyrosine kinase-type cell surface receptor HER2 (HER2, receptor tyrosine-protein kinase erbB-2, p185erbB2, c-ErbB2, NEU proto-oncogene [neu], MLN 19, CD340)

is the most oncogenic, as it is active in the absence of a stimulating ligand. In addition, HER2 in heterodimeric receptors is highly mitogenic because it decelerates growth factor dissociation from its partner receptor, prolonging the duration of intracellular signaling. In addition, endocytosis of HER2-containing complexes is relatively slow and these complexes tend to recycle back to the cell surface, rather than being degraded in the lysosomes. HER2, a 185 kDa transmembrane glycoprotein receptor, underlies many altered functions of tumor cells, including excessive growth, invasive behavior and attraction of blood vessels (Mosesson and Yarden, 2004). It is overexpressed in 25% to 30% of breast cancers, and it has been associated with high risks of relapse and death (Slamon *et al.*, 1987), although the prognostic value of HER2 has been the matter of some controversy (Ross *et al.*, 2003). Blockage of HER2 with the humanized anti-HER2 monoclonal antibody trastuzumab is an example of successful immunotherapy for HER-2-positive breast cancer patients, as it has been demonstrated to reduce the risk of recurrence by roughly 50% in five randomized clinical trials (Piccart-Gebhart, 2006).

The HER2 ectodomain is released and detected in the serum of cancer patients. High serum levels of HER2 ectodomain correlate with a poor prognosis and decreased responsiveness to therapy in patients with advanced breast cancer. This may be due to the enhanced signaling activity of the remaining cell-associated part of HER2. The cleavage is inhibited by the metalloproteinase inhibitors batimastat and TNF-α protease inhibitor (TAPI), as well as by TIMP-1, which inhibits soluble MMPs (Codony-Servat *et al.*, 1999). In addition, trastuzumab inhibits basal and induced HER2 cleavage, preceding antibody-induced receptor downmodulation (Molina *et al.*, 2001). Further identification of the metalloproteinase in charge could lead to new options for the therapy of patiens with breast cancer and high levels of HER2 shedding, for example by combining MMP inhibition with trastuzumab therapy.

1.2 Survival of Cancer Cells or Induction of Apoptosis

Evasion of apoptosis permits survival of tumor cells in spite of genetic instability. Low levels of oxygen and nutrients, host defence against the tumor by the immune system, anti-cancer treatments and local *in vivo* changes in the ECM with effects on invasion and metastasis alter the protease load in the tumor environment (Reed, 1999). Proteolysis by MMPs can have apoptotic as well as anti-apoptotic effects.

1.2.1 Fas Ligand (FasL)

An important effector in apoptosis is the Fas/FasL system, which is involved in three types of immune-associated killing: 1) elimination of virally infected cells and tumor cells by cytotoxic T cells (CTLs) and natural killer (NK) cells; 2) maintenance of immune privilege or survival of tumor cells; and 3) regulation of lymphocyte development and maintenance of peripheral immune homeostasis. Fas ligand (FasL, tumor necrosis factor ligand superfamily member 6, CD95 L, apoptosis antigen ligand (APTL), APO-1 L, CD178) is a ~40 kDa type II transmembrane protein of the TNF family of death factors. It is expressed on activated T lymphocytes like CTLs and tumor infiltrating lymphocytes (TILs), on macrophages and NK cells and on cells within immune privileged tissues including the eye, testis, uterus, and placenta (Linkermann *et al.*, 2003). After trimerization FasL induces apoptosis by binding to a trimer of its receptor Fas (tumor necrosis factor receptor superfamily member 6, apoptosis-mediating surface antigen FAS, APO-1, FASLG receptor, CD95). FasL can be released form the cell surface by MMP-3 and MMP-7. However, the effects of soluble FasL (sFasL) on apoptosis and tumor progression seem to vary. Apoptosis may be induced in epithelial cells through cleavage of membrane-bound FasL to functional sFasL by MMP-7. Furthermore, this cleavage turns out to be crucial for apoptosis of prostate epithelium after castration, as prostate involution is significantly reduced in MMP-7 deficient mice (Powell *et al.*, 1999). In contrast with apoptosis induction in epithelial cells, FasL shedding by MMP-7 has an anti-apoptotic effect in tumor cells, protecting them from chemotherapeutic drug toxicity (Mitsiades *et al.*, 2001). This protection of tumor cells may be explained by the fact that the ability of sFasL to induce apoptosis is significantly lower than that of its cell surface precursor (Tanaka *et al.*, 1998). A possible explanation for the paradoxical effects of FasL in both cell types might be the higher sensivity of epithelial cells to apoptotic signals, while FasL confers a kind of immune privilege to tumors by inducing apoptosis in infiltrating lymphocytes. Tumor cells indeed express lower levels of Fas and increased levels of FasL, which allows them to 'counterattack' Fas-bearing immune cells (Kim *et al.*, 2004). Acute overexpression of MMP-7 *in vitro*

as well as *in vivo* induces apoptosis, whereas chronic or repeated exposure to MMP-7 can select for tumor cells that are less sensitive to death-inducing stimuli (Fingleton *et al.*, 2001; Vargo-Gogola *et al.*, 2002b). Another potential explanation for the mentioned discrepancies in sFasL activity came up after the identification of novel MMP-7 cleavage sites in murine and human FasL. Mutational analysis showed that MMP-7 cleaves human and murine FasL at an ELAELR sequence, close to the transmembrane domain (Figure 5), followed by a secondary cleavage at the COOH-terminally located SL sites. Strong indications exist that cleavage of human FasL also occurs at another ELR sequence in the trimerization domain. As trimerization of FasL has been shown to be important for its activity, cleavage within the trimerization domain may play a significant role in the regulation of sFasL function, releasing less active FasL. Human sFasL peptides generated after MMP-7 cleavage at ELAELR contain 13 or 16 additional amino acids compared to the peptides formed after proteolysis at the SL site. The entire extracellular portion of FasL was shown to be pro-apoptotic *in vitro*, whereas sFasL produced by cleavage at the SL site was inactive in the same assay (Hohlbaum *et al.*, 2000). This implies that the amino acid sequence between the SL cleavage site and the transmembrane domain contributes to the pro-apoptotic activity of sFasL. Therefore, truncation at the SL site might release inactive sFasL peptides (Vargo-Gogola *et al.*, 2002a). Differential FasL proteolysis by MMP-7 might thus lead to the release of sFasL molecules with diverse biological activities.

1.2.2 Fas

MMP-7 expression also contributes to another strategy by which tumor cells can resist Fas-induced apoptosis. As a matter of fact, Fas too is cleaved by MMP-7, between Glu19-Leu20 and Asn32-Leu33 (Figure 6) (Strand *et al.*, 2004). These cleavages remove 19 or 32 amino acids from the extracellular NH_2-terminus of the Fas molecule and delete part of a domain for self-association termed 'preligand assembly domain' (PLAD). The PLAD domain has been shown to facilitate oligomerization of Fas receptors before ligand binding. Preassembly of Fas receptors might be crucial for the regulation of Fas signaling (Siegel *et al.*, 2000). Fas proteolysis by MMP-7 indeed results in attenuated apoptosis induction (Strand *et al.*, 2004). MMP-7 is produced by the tumor cells themselves at early stages of tumor development. Therefore, the impact of this MMP on apoptosis resistance provides a potential target for new combination therapies in which MMP inhibition may significantly augment the efficacy of conventional chemotherapy (Poulaki *et al.*, 2001).

1.3 Regulation of Angiogenesis

Oxygen and nutrients, provided by the vascular system, are crucial for cell function and survival, obligating virtually all cells in a tissue to reside within 100 μm of a capillary blood vessel. Consequently, angiogenesis or the formation of new blood vessels, is a prerequisite for the survival of proliferating cells and is the result of subtle and often complex interactions between regulator and effector molecules (Hanahan and Weinberg, 2000; Pepper, 2001; Bergers and Benjamin, 2003).

1.3.1 Urokinase-Type Plasminogen Activator Receptor (uPAR)

The urokinase-type plasminogen activator receptor (uPAR, monocyte activation antigen Mo3, CD87) is a GPI-anchored receptor containing three homologous domains (D1, D2, and D3) (Blasi and Carmeliet, 2002). D1 is involved in the binding of the urokinase-type plasminogen activator (urokinase or u-PA) and enhances the interaction with the ECM by binding the ECM molecule vitronectin. D2 and D3 are also indispensable for high-affinity interactions (Hoyer-Hansen *et al.*, 1997a; Oda *et al.*, 1998). uPAR regulates u-PA activity–the activation of plasminogen to plasmin that degrades fibrin (Blasi and Carmeliet, 2002)–on the cell surface and is also important for the activation of signaling pathways through the interaction with several integrins (Reuning *et al.*, 2003). Besides the membrane-anchored uPAR, a soluble receptor (suPAR) is released after proteolysis of the GPI anchor by cellular phospholipase D (Figure 7) (Wilhelm *et al.*, 1999). Both uPAR and suPAR can be cleaved between the D1 and D2 domain, generating a D1-fragment and a D2D3-fragment (Blasi and Carmeliet, 2002). The D1 domain can be released by proteolytic activity of u-PA, directly or indirectly through activation of plasminogen (Hoyer-Hansen *et al.*, 1997b), or by other proteases such as chymotrypsin and elastase (Ploug and Ellis, 1994). The specificty of the cleaving protease is crucial, as uPAR fragments may or may not be chemotactically active on different cell types *in vitro*, depending on the presence or

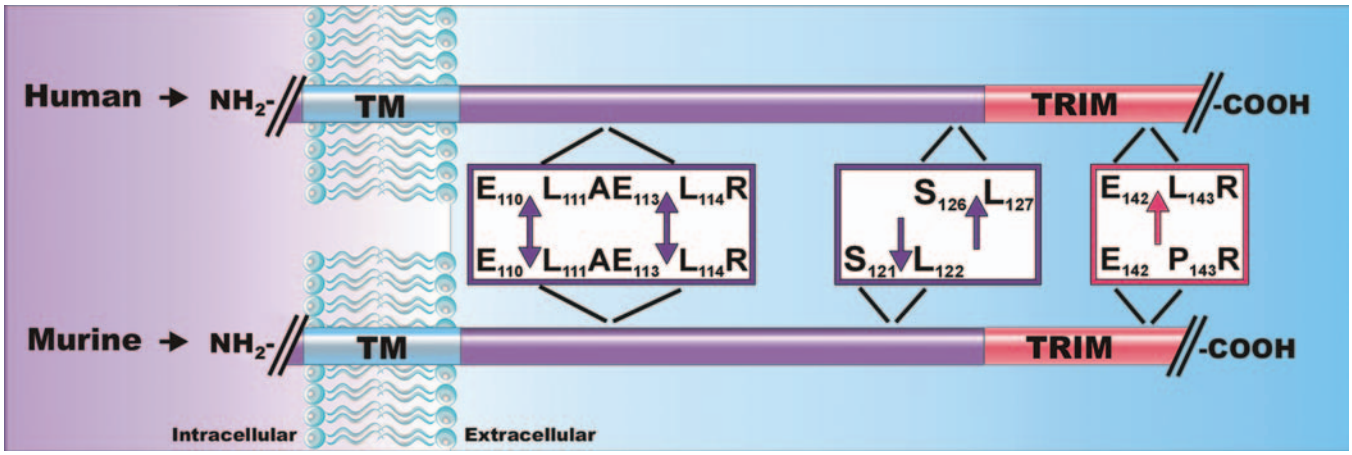

FIGURE 5 **Cleavage sites of MMP-7 in the ectodomain of human and murine FasL. MMP-7 cleaves human and murine FasL at an ELAELR sequence, close to the transmembrane domain (TM), followed by a secondary cleavage at COOH-terminally located SL sites. Human sFasL peptides generated after MMP-7 cleavage at ELAELR were shown to be pro-apoptotic *in vitro*. However, the peptides formed after proteolysis at the S_{126}-L_{127} site, which contain 13 or 16 amino acids less, were inactive in the same apoptosis assay. This might mean that the amino acid sequence between the SL cleavage site and the transmembrane domain contributes to the pro-apoptotic activity of sFasL. Cleavage of human FasL almost certainly occurs at an additional ELR sequence, E_{142}-L_{143}R, in the trimerization domain (TRIM). As trimerization of FasL has been shown to be important for its activity, cleavage within the trimerization domain may release less active FasL. Arrows indicate the cleavage sites of MMP-7. Adapted from (Vargo-Gogola *et al.*, 2002a).**

absence of a specific NH_2-terminal peptide, SRSRY, on the D2D3-fragment (Fazioli *et al.*, 1997). Inhibition of MMPs in a three-dimensional fibrin matrix, used as an experimental angiogenesis model, caused enhanced formation of capillary-like tubular structures and showed that the cleavage between D1 and D2 can also be performed by MMPs. The first MMP to be identified as uPAR sheddase was MMP-12 (Koolwijk *et al.*, 2001), but also several other MMPs were able to release the D1 domain of suPAR *in vitro*: MMP-2, MMP-3, MMP-8, MMP-9, MMP-13, MMP-19, MT1-MMP, and MT6-MMP (Andolfo *et al.*, 2002). However, the most efficient MMPs for uPAR cleavage were MMP-3, MMP-12, MMP-19, and MT6-MMP. In particular, MMP-3, MMP-12, and MT6-MMP show the same specificity of cleavage having the Thr86-Tyr87 peptide bond as major cleavage site, while MMP-19 cleaves suPAR predominantly at Tyr87-Ser88 (Figure 7). uPAR cleavage by MMPs results in the exposure of the chemotactic epitope SRSRY at the NH_2-terminus of the generated D2D3-fragment and may thus generate biologically active fragments. Murine uPAR is cleaved by MMP-12

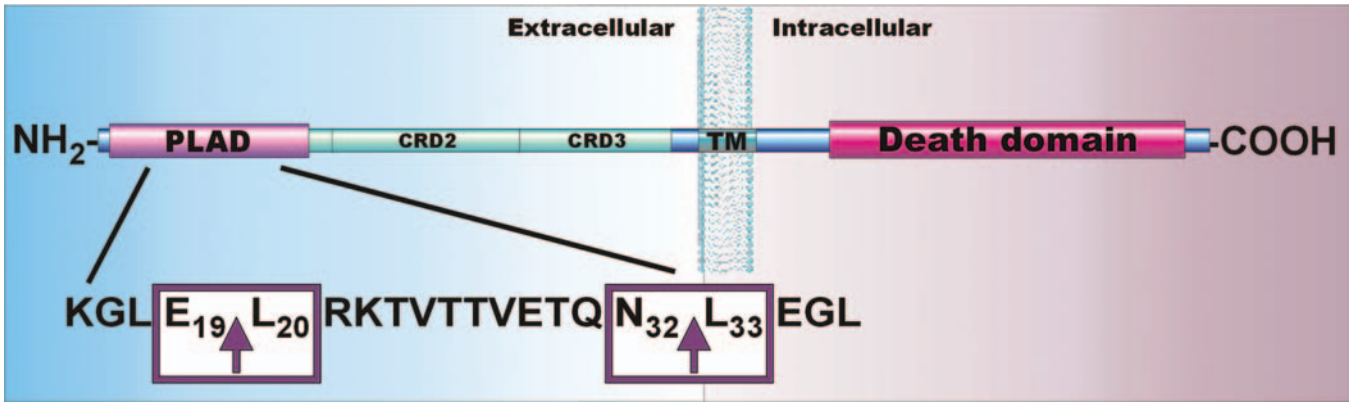

FIGURE 6 **MMP-7 cleaves the 'preligand assembly domain' (PLAD) in the Fas ectodomain. Fas is cleaved by MMP-7 between Glu19-Leu20 and Asn32-Leu33. These cleavages remove 19 or 32 amino acids from the Fas NH_2-terminus and delete part of a domain for self-association termed 'preligand assembly domain' (PLAD). The PLAD domain facilitates oligomerization of Fas receptors before ligand binding. Preassembly of Fas receptors may be crucial for the regulation of Fas signaling and proteolysis of the PLAD domain by MMP-7 indeed results in decreased sensitivity of tumor cells to Fas-mediated apoptosis. Arrows indicate the cleavage sites of MMP-7; CRD, cysteine-rich domain; TM, transmembrane domain. Adapted from (Strand *et al.*, 2004).**

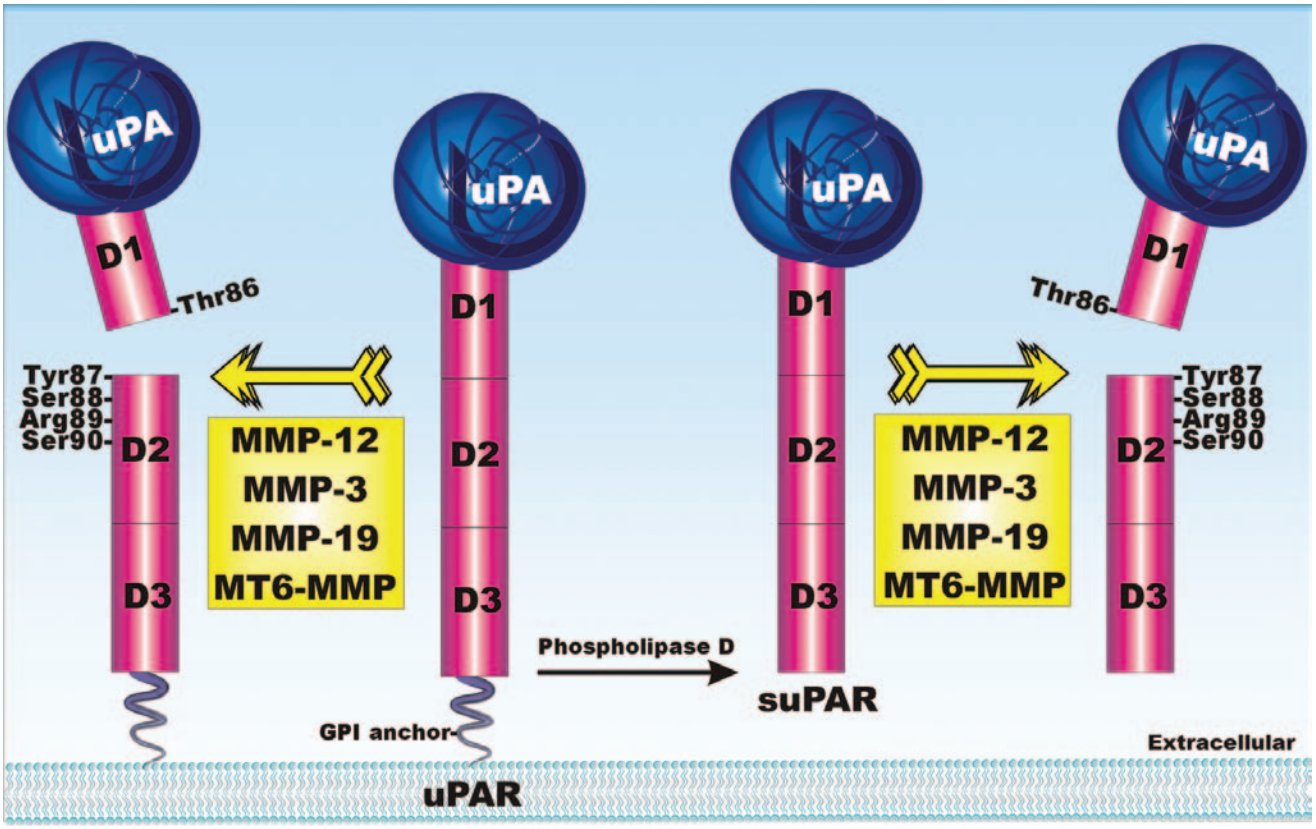

FIGURE 7 **Cleavage of uPAR and suPAR by MMPs releases the u-PA-binding domain D1. uPAR is a GPI-anchored receptor for u-PA containing 3 homologous domains (D1, D2, and D3). In addition to the membrane-anchored uPAR, a soluble receptor (suPAR) is released after cleavage of the GPI anchor by cellular phospholipase D. Both uPAR and suPAR can be cleaved between the D1 and D2 domains, generating a D1-fragment and a D2D3-fragment. Release of the D1 domain can be mediated by MMPs, but also by serine proteases such as u-PA, plasmin, chymotrypsin and elastase. The first MMP to be identified as uPAR sheddase was MMP-12 but also several other MMPs release the D1 domain of suPAR *in vitro*. However, the MMPs most efficient at uPAR cleavage are MMP-3, MMP-12, MMP-19 and MT6-MMP. In particular, MMP-3, MMP-12 and MT6-MMP show the same specificity of cleavage with the Thr86-Tyr87 peptide bond as major cleavage site, whereas MMP-19 cleaves suPAR predominantly at Tyr87-Ser88. Loss of the D1 domain results in less cellular u-PA binding and, consequently, in diminished formation of new capillary structures in an angiogenesis model.**

as well (at the Pro89-Gln90 peptide bond), despite the limited sequence homology between the linker regions (Andolfo *et al.*, 2002).

Unexpected enhanced angiogenesis through MMP inhibition might be caused by the higher availability of functional uPAR at the cell surface, resulting in increased u-PA binding and, subsequently, enhanced formation of new capillary structures. As plasmin activates several MMPs (for reviews, see: Collen, 2001; Pepper, 2001; Van den Steen *et al.*, 2001), release of the D1 fragment by an MMP might be a kind of feedback regulation. Since angiogenesis in and growth of some tumors are inhibited by competitors of uPAR, and taking into account that some MMPs (such as MMP-9) exert pro-angiogenic effects, the influence of MMP inhibition on uPAR levels and on angiogenesis certainly requires further examination.

Cleavage of uPAR by MMP-12 may also play a role in the autoimmune disease systemic sclerosis. This will be detailed in a subsequent paragraph (see Section 3.4.4).

1.3.2 Betaglycan

Betaglycan, also known as the TGF-β type III receptor, is a membrane-anchored proteoglycan whose glycosaminoglycan chains consist of heparan and chondroitin sulfate. Membrane-bound betaglycan is generally considered a positive regulator of TGF-β because it increases the binding affinity of TGF-β for its receptor II, enhancing cell responsiveness to TGF-β (Lopez-Casillas *et al.*, 1994). TGF-β controls many physiological processes and has tumor-suppressing activity in the early phases of carcinogenesis. In subsequent stages of tumor progression, the increased secretion of TGF-β by both tumor cells and stroma cells, is involved in the

enhancement of tumor invasion and mestastasis, accompanied by immunosuppression (Kim *et al.*, 2004). TGF-β upregulates MMP-9 activity, which may also amplify angiogenesis and tumor growth. Additionally, MMP-9 is capable of activating latent TGF-β (Yu and Stamenkovic, 2000; Bandyopadhyay *et al.*, 2005), thanks to the anchoring of MMP-9 and TGF-β to CD44 on the cell surface (see Section 1.4.7) (Yu and Stamenkovic, 1999).

Two soluble forms of betaglycan are relased by proteolytic cleavage. In some cell types these cleavages are induced by the tyrosine phosphatase inhibitor, pervanadate, and generate a bigger fragment of 120 kDa (sBG-120), which encompasses almost the entire extracellular domain, and a smaller 90 kDa fragment (sBG-90). The cleavage that generates sBG-90 is inhibited by TIMP-2, but not by TIMP-1, which points to an MT-MMP as the involved protease. Overexpression of MT1-MMP and MT3-MMP, but not of the other MT-MMPs, indeed releases the sBG-90 fragment. Surprisingly, MT2-MMP overexpression decreases the levels of betaglycan and of MT1-MMP (Velasco-Loyden *et al.*, 2004). In contrast with membrane-bound betaglycan, recombinant soluble betaglycan has been shown to inhibit TGF-β *in vitro* (Lopez-Casillas *et al.*, 1994). Therefore, betaglycan might function as a dual modulator of TGF-β activity: as a membrane-anchored protein it enhances TGF-β activity, whereas its soluble form causes TGF-β inhibition. TGF-β-promoted tumor-host interactions leading to enhanced angiogenesis have been shown to be effectively attenuated by the systemic administration of soluble betaglycan in a xenograft model of prostate cancer. The inhibition of tumor angiogenesis and consequently of tumor growth appears at least in part due to the inhibition of TGF-β-induced MMP-9 upregulation (Bandyopadhyay *et al.*, 2005). Further investigation will clarify if sBG-90 and sBG-120 have the same TGF-β-inhibiting activities and if proteolysis of betaglycan by MT1-MMP or MT3-MMP can reduce angiogenesis and tumor growth.

1.3.3 Vascular Endothelial Cadherin (VE-Cadherin)

Vascular endothelial-cadherin (VE-cadherin, cadherin-5, 7B4 antigen, CD144) is a member of the large cadherin family that includes Ca^{2+}-dependent cell-cell adhesion molecules responsible for cell-to-cell recognition and adhesion in solid tissues. Cadherins dimerize through the extracellular domain with other cadherin molecules on adjacent cells (*trans*-interaction). In this homotypic interaction, the intracellular domain interacts with various catenin proteins to form the cytoplasmic cell-adhesion complex (CCC), which is crucial for strong cell-cell adhesion and potent suppression of invasion. Cadherins are expressed in several types of tissues with some specificity: Epithelial (E)-cadherin is mostly present in epithelial cells, Neuronal (N)-cadherin in the nervous system, smooth muscle cells, fibroblasts and endothelial cells, and VE-cadherin is specific for the endothelium (Cavallaro and Christofori, 2004; Cavallaro *et al.*, 2006).

MMP-7 treatment of human umbilical endothelial cells (HUVECs) accelerates HUVEC proliferation and degrades VE-cadherin on the cell surface, with concomitant accumulation of β-catenin in the nucleus and an increase of MMP-7 expression. These results suggest that MMP-7-mediated cleavage of VE-cadherin releases β-catenin from the VE-cadherin/catenin complex, allowing it to translocate from the cytoplasm to the nucleus, where it can activate T-cell factor DNA binding protein, which accelerates cell proliferation and MMP-7 expression (Ichikawa *et al.*, 2006). However, MMP-7 also has another important role in angiogenesis as it cleaves plasminogen and converts it to angiostatin (Patterson and Sang, 1997), which is one of the strongest inhibitors of angiogenesis. In light of this, it is crucial to further examine the sometimes paradoxical effects of MMP-7 on angiogenesis before targetting its activity in anti-cancer therapy.

1.3.4 Semaphorin 4D

Semaphorins are secreted, transmembrane or GPI-linked proteins, defined by cysteine-rich semaphorin protein domains, that have essential roles in a variety of tissues. Functionally, semaphorins were initially characterized for their importance in the development of the nervous system and in axonal guidance. More recently, they have been found to play a role in a wide range of processes, including tissue organization during development, angiogenesis, immunoregulation, and tumor progression. A common theme in the mechanisms of semaphorin function is that they alter the cytoskeleton, *i.e.*, the organization of actin filaments and the microtubular network, through binding with their receptors. The best characterized semaphorin receptors are members of the neurophilin and plexin families (Yazdani and Terman, 2006). Plexin-1B is highly expressed in endothelial cells and promotes migration and

tubulogenesis *in vitro* as well as *in vivo* when bound by its ligand, semaphorin 4D (BB18, A8, GR3, CD100) (Basile *et al.*, 2004). Semaphorin 4D is highly expressed in head and neck squamous cell carcinomas as well as in some of the most prevalent solid tumors, including breast, prostate, colon and lung cancer tissues (Basile *et al.*, 2006).

In order to exert its pro-angiogenic functions, semaphorin 4D, a transmembrane protein, must be processed and released into a soluble form to act in a paracrine manner on endothelial cells. Semaphorin 4D is expressed on the cell surface as a homodimer (see Figure 2), which is a prerequisite for its proteolytic release from the cell surface (Elhabazi *et al.*, 2001). The shedding process can be inhibited by the metalloproteinase inhibitors EDTA, EGTA and Ilomastat/GM6001, as well as by TIMP-2, but not by TIMP-1 (Elhabazi *et al.*, 2001; Basile *et al.*, 2007). The inhibitor profile, combined with the observation that MT1-MMP, while not expressed in non-tumorigenic epithelial cell lines, was present in several head and neck squamous carcinoma cell lines, pointed to MT1-MMP as the semaphorin 4D sheddase. Basile and coworkers (2007) demonstrated that MT1-MMP was required for processing and release of semaphorin 4D from these cells, thereby inducing endothelial cell chemotaxis *in vitro* and blood vessel growth *in vivo*. As a consequence, MT1-MMP-dependent shedding of semaphorin 4D may play a critical role in tumor-induced angiogenesis, and therefore may represent new fronts of attack in the anti-angiogenic therapy of cancer.

1.4 Stimulation or Inhibition of Migration, Invasion, and Metastasis

Much like tumorigenesis, the processes of invasion and metastasis are highly complex. In its simplest form, metastasis requires the tumor cell to detach from its primary location, invade through stromal elements or existing junctions between normal cells, enter and leave blood vessels or lymphatics, and then establish a colony at the metastatic site. These steps require molecular processes at the cell surface in which contacts between the invading tumor cell and surrounding cells and stroma are repeatedly broken (anti-adhesion) and new contacts established as the tumor cell moves forward (adhesion). This alternation of adhesion and anti-adhesion can be achieved through coordinated expression of proteases and adhesion molecules (Chambers *et al.*, 2002; Hollingsworth and Swanson, 2004).

1.4.1 Mucin-1 (MUC1)

Mucin-1 (MUC1, polymorphic epithelial mucin [PEM, PEMT], episialin, tumor-associated mucin, carcinoma-associated mucin, tumor-associated epithelial membrane antigen [EMA], H23AG, peanut-reactive urinary mucin [PUM], breast carcinoma-associated antigen DF3, CD227), a transmembrane mucin, plays a key role in the inhibition of embryo implantation, in the protection of mucosal surfaces against microbial and proteolytic degradation, and in some aspects of tumor progression. It is expressed on several epithelial surfaces like uterine, lung and intestinal epithelia as well as on tumor cells. The relatively short cytoplasmic tail associates with cytoskeletal elements, cytosolic adaptor proteins and/or participates in signal transduction. The extracellular domain can be released form the cell surface, which might serve practical functions, such as the facilitation of rapid clearance of mucosa surface-associated material, or cell mobility. The mechanism controling this ectodomain release has not yet been elucidated, even though it has been postulated that alterations in pH, ionic concentration or hydration might trigger proteolytic release by specific proteases (Hollingsworth and Swanson, 2004). The major protease in this cleavage process is TACE (Thathiah *et al.*, 2003). However, according to the TIMP-inhibition profile (see Table 1), an additional MUC1 shedding activity belonging to the MT-MMP family was identified in TACE deficient cells. MT1-MMP is expressed on these cells and, in addition, MT1-MMP overexpression or deficiency causes increased or inhibited MUC1 shedding, respectively. Furthermore, MT1-MMP indeed cleaves MUC1 *in vitro* and is colocalized with MUC1 *in vivo* in human uterine epithelia (Thathiah and Carson, 2004). Cancer cells, especially from adenocarcinomas, express aberrant forms and levels of mucins, which have an impact on the biological properties of tumors in several ways (Hollingsworth and Swanson, 2004). Evidences are emerging that cell surface mucins contribute to the regulation of differentiation and proliferation of tumor cells, through ligand-receptor interactions and morphogenetic signal transduction. In addition, MUC1 expression on tumor cells causes anti-adhesion through steric hindrance by forming multiple exposed glycosylated rod-like structures and by the binding of receptors on the same cell (*cis*-interactions), preventing

interaction of these receptors with other cells. This anti-adhesion permits tumor cells to detach form the tumor mass and to invade the surrounding stroma. Invasion is subsequently enhanced, as MUC1 also has an adhesive action by binding adhesion molecules on stroma cells and endothelial cells (*trans*-interactions). Finally, MUC1 also contributes to immune evasion by forming a leukocyte-impermeable barrier around the tumor and through immunosuppressive effects on T-cell proliferative responses. Since MT1-MMP is often expressed by cancer cells, the cleavage of MUC1 may affect all these processes.

1.4.2 Epithelial Cadherin (E-Cadherin)

Epithelial cadherin (E-cadherin, uvomorulin, cadherin-1, CAM 120/80, CD324) is another member of the cadherin family and is expressed mostly in epithelial cells (see Section 1.3.3). Loss of E-cadherin-mediated cell-cell adhesion has been shown to be a prerequisite for tumor cell invasion and metastasis (Birchmeier and Behrens, 1994). Proteolytic degradation of E-cadherin by MMPs is one of the regulation mechanisms by which epithelial cell-cell adhesion can be ablated. MMP-3 and MMP-7 both cleave the 120 kDa transmembrane E-cadherin, releasing a 80 kDa soluble form (sE-cadherin) (Lochter *et al.*, 1997; Noe *et al.*, 2001). Treatment of prostate cancer cells with hepatocyte growth factor/scatter factor (HGF/SF) causes shedding of E-cadherin through the induction of MMP-7, resulting in cell scattering and a switch to a more invasive phenotype (Davies *et al.*, 2001). Released sE-cadherin induces invasion *in vitro* and inhibits cell aggregation indicating that it disturbs cell-bound E-cadherin functions in a paracrine way (Noe *et al.*, 2001). However, it is not clear whether sE-cadherin induces invasion by perturbation of cell-cell interaction and/or by engaging a signalling pathway in which free β-catenin can act as a transcriptional coactivator (Hecht and Kemler, 2000). An induction of MMP-2, MMP-9, and MT1-MMP expression was observed both at the mRNA and protein levels in the presence of sE-cadherin. ECM degradation by these MMPs might be an extra mechanism by which E-cadherin ectodomain shedding contributes to tumor invasion and metastasis formation (Nawrocki-Raby *et al.*, 2003). Besides the influence on invasion and metastasis, proteolysis of E-cadherin also plays a role in epithelial-mesenchymal transition, a conversion to an altered cellular phenotype which is associated with aggressive malignant behaviour (Cavallaro and Christofori, 2004). Induction of MMP-3 expression in mammary epithelial cells results in E-cadherin cleavage and triggers a progressive phenotypic conversion cumulating in cells that are unable to undergo lactogenic differentiation and that become invasive (Lochter *et al.*, 1997; Sternlicht *et al.*, 1999). Finally, during apoptosis, the cytoplasmic tail of E-cadherin is truncated by caspase-3, whilst a 84 kDa ectodomain fragment is released by a metalloproteinase. This simultanous cleavage of intracellular and extracellular domains might be a highly efficient mechanism to disrupt E-cadherin-dependent cell-cell contacts in apoptotic cells or tumor cells, which is a prerequisite for cell rounding and exit from the epithelium (Steinhusen *et al.*, 2001).

In acute renal failure, E-cadherin degradation by MT1-MMP leads to disruption of epithelial integrity and epithelial cell shedding (see Section 2.2.3), whereas in normal physiology, MMP-7-mediated shedding of E-cadherin is required for the repair of injured lung epithelium (McGuire *et al.*, 2003).

1.4.3 Integrin Subunit Precursors

Integrins are a diverse family of transmembrane glycoproteins that form heterodimeric receptors for ECM molecules and membrane-associated molecules of the Ig family. Every integrin is composed of a non-covalently coupled α- and β-subunit. The 18 known α-subunits and 8 β-subunits form at least 25 distinct heterodimers in human, with each pair being specific for a unique set of ligands. Integrins are crucial for cell adhesion, migration and invasion, not only through the direct physical adhesion to the ECM and to other cells, but also because they send and receive molecular signals that are essential for these processes (inside-out and outside-in signaling, respectively) (Hood and Cheresh, 2002).

The binding of integrins to ECM molecules is altered by changes in integrin expression and affinity when cancer cells become metastatic, or when endothelial cells enter the angiogenic state (Varner and Cheresh, 1996; Demetriou and Cress, 2004). Maturation of some integrin subunits requires a posttranslational cleavage of the precursor chain. The exact role of this endoproteolytical modification in integrin function is unclear, but its absence has important consequences for signal transduction pathways and leads to alterations in integrin functions such as cell adhesion to vitronectin (Berthet *et al.*, 2000). Proteolysis of pro-integrin chains is performed by proprotein convertases (PC) of the subtilisin/

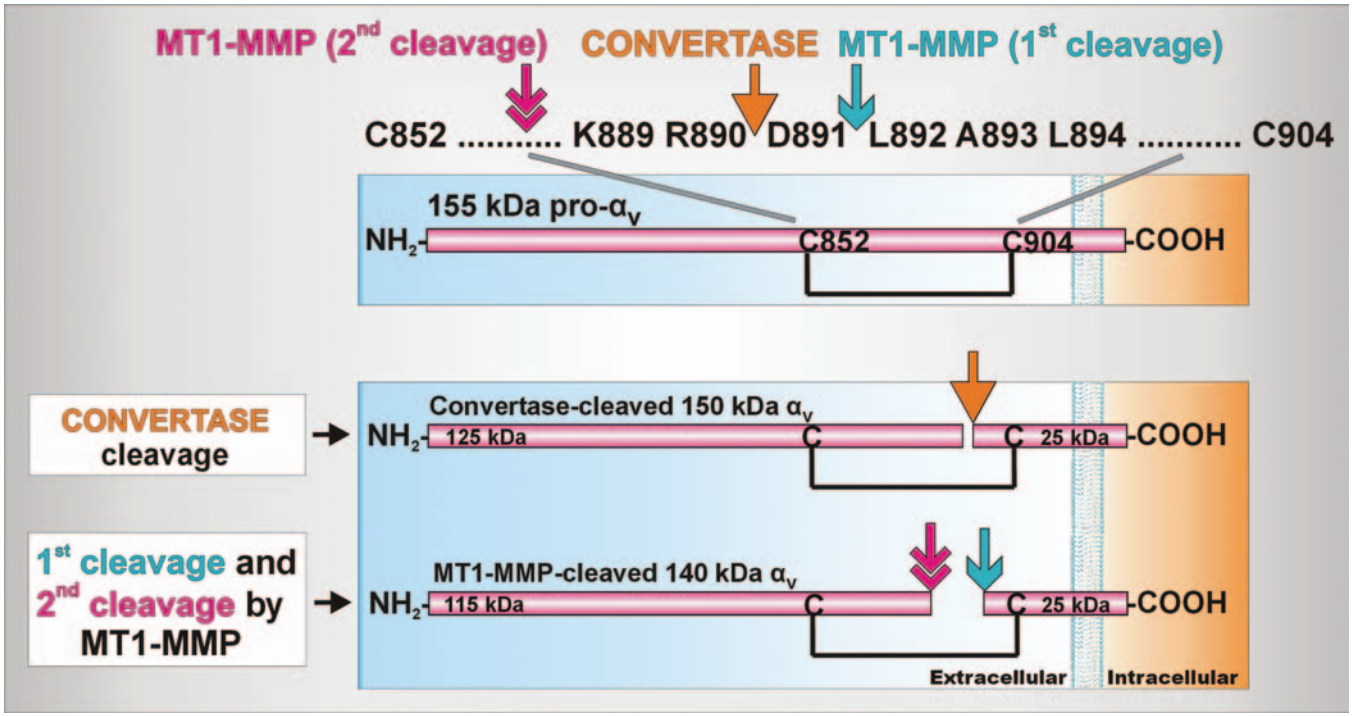

FIGURE 8 **Endoproteolytic cleavage of pro-α_v-integrin by MT1-MMP and by a proprotein convertase. Proteolysis of pro-integrin chains is performed by proprotein convertases, but also by MT1-MMP. MT1-MMP cleaves the pro-α_v chain at two different positions between the disulfide-connected Cys852 and Cys904. The first cleavage, between Asp891 and Leu892, generates a 125 kDa heavy α-chain, disulfide-bound to a 25 kDa light chain that is one residu shorter at the N-terminus in comparison with the convertase-processed chain. The second MT1-MMP cleavage removes an additional 10 kDa from the heavy α-chain and is situated downstream from the Cys852. These proteolytic modifications do not affect ligand binding of the resulting $\alpha_v\beta_3$ integrin but enhance outside-in signal transduction, which results in more efficient adhesion and migration on vitronectin. Adapted from (Ratnikov *et al.*, 2002).**

kexin-like family, but also by MT1-MMP. MT1-MMP cleaves the pro-α_v chain (integrin α-V precursor, vitronectin receptor subunit α, CD51) at two different positions between the disulfide-connected Cys852 and Cys904. The first cleavage, between Asp891 and Leu892, generates the 125 kDa heavy chain, disulfide-bound to a 25 kDa light chain that is one residu shorter at the N-terminus in comparison with the PC-processed chain (Figure 8). The putative second MT1-MMP cleavage site is situated downstream from the Cys852 and produces a 115 kDa heavy α-chain (Ratnikov *et al.*, 2002). These proteolytic modifications do not affect ligand binding of the resulting $\alpha_v\beta_3$ integrin but enhance outside-in signal transduction. As a result, cells co-expressing MT1-MMP and $\alpha_v\beta_3$ integrin show more efficient adhesion and migration on vitronectin, the ECM ligand of $\alpha_v\beta_3$ (Deryugina *et al.*, 2002).

In addition, the MT1-MMP-mediated cleavage seems to regulate cross-talk between $\alpha_v\beta_3$ and $\alpha_2\beta_1$ integrins. In MT1-MMP deficient cells, the functional activity of the collagen-binding $\alpha_2\beta_1$-integrin, which is not cleaved by MT1-MMP, is suppressed by the presence of $\alpha_v\beta_3$ integrin, resulting in diminished cell adhesion to collagen type I. Co-expression of MT1-MMP and $\alpha_v\beta_3$ restores the $\alpha_2\beta_1$-mediated collagen binding. Expression of both $\alpha_v\beta_3$ integrin and MT1-MMP is elevated in malignant tumor cells and is correlated with increased migration. Cross-talk between $\alpha_v\beta_3$ and $\alpha_2\beta_1$ integrins through MT1-MMP-mediated pro-α_v-proteolysis might thus contribute to efficient adhesion of aggressive tumor cells to type I collagen, an important substratum of the ECM (Baciu *et al.*, 2003). In addition, MT1-MMP modifies the β_3-subunit (integrin β-3 precursor, platelet membrane glycoprotein IIIa (GPIIIa), CD61) of $\alpha_v\beta_3$ integrin from a 95 kDa to a 90 kDa chain, which is also correlated with functional $\alpha_v\beta_3$ integrin activation and increased adhesion on vitronectin (Deryugina *et al.*, 2000). Moreover, after functional activation by MT1-MMP, $\alpha_v\beta_3$ integrin shows a higher affinity for the MMP-2 hemopexin domain, and binding to $\alpha_v\beta_3$ integrin concentrates MMP-2 at specific spots on the cell surface, which may again contribute to cell migration through the ECM. Hence, functional regulation of integrins by MT1-MMP plays an essential role in

efficient adhesion and directional migration of tumor cells. As a consequence, this pathway could be another interesting target in therapeutic approaches aiming at suppressing tumor cell spreading.

MT1-MMP also mediates maturation of the pro-α_3 (integrin α-3 precursor, galactoprotein B3 (GAPB3), VLA-3 α chain, FRP-2, CD49c) and pro-α_5 (integrin α-5 precursor, fibronectin receptor subunit α, integrin α-F, VLA-5, CD49e) chains to the respective mature disulfide-bound heavy and light α-chains (Baciu *et al.*, 2003). However, pro-α_5 cleavage does not affect $\alpha_5\beta_1$ integrin function. The consequences of pro-α_3 cleavage have not been investigated.

MMP-7 is another tumor-derived MMP capable of integrin subunit proteolysis. MMP-7 releases a 90 kDa fragment of the 200 kDa β_4-subunit precursor (integrin β-4 precursor, GP150, CD104), possibly through cleavage of the Tyr106-Ile107 and Gly416-Leu417 peptide bounds. The first putative cleavage site is very close to the ligand-binding domain, and cleavage might thus interfere with the binding of the $\alpha_6\beta_4$ integrin to its ligand laminin (von Bredow *et al.*, 1997). $\alpha_6\beta_4$ integrin has been most implicated in epithelial carcinogenesis (Watt, 2002). In epithelium-derived carcinoma, increased levels of β_4 integrin and loss of its polarized distribution to the basolateral membrane site of the cell have been correlated with tumor aggressiveness (Rigot *et al.*, 1999). Adhesion of breast cancer cells to endothelial cells through binding of $\alpha_6\beta_4$ integrin to a specific lung-endothelial cell adhesion molecule is critical for lung metastasis, but is totally abolished after cleavage of the β_4 integrin ectodomain by MMP-7 (Abdel-Ghany *et al.*, 2001). Hence, in this case, MMP-mediated modification of an integrin subunit reduces tumor cell adhesion and migration.

Furthermore, integrins are important players in tumor cell proliferation, apoptosis and angiogenesis, in leukocyte migration and in a whole array of pathologies (Wehrle-Haller and Imhof, 2003). As a consequence, insight in the functional regulation of these bidirectional signaling molecules by MMPs may generate new possibilities for therapeutic intervention.

1.4.4 Tissue Transglutaminase (tTG)

Another adhesion and signaling receptor being cleaved by MT-MMPs is the ubiquitously expressed cell surface-associated tissue transglutaminase (tTG, protein-glutamine γ-glutamyltransferase 2, TGase C (TGC), transglutaminase-2, TGase-H). tTG catalyzes covalent cross-linking between reactive lysine and glutamine residues of proteins and protein polymers. In addition, tTG functions as a coreceptor for β_1 and β_3 integrins and promotes integrin-dependent adhesion and cell spreading on fibronectin. Good functioning of cell surface tTG is essential for regulation and maintenance of cell-matrix interactions, as well as for the mobility of tumor and host cells. On the contrary, deregulation of tTG activity is associated with multiple human diseases (Griffin *et al.*, 2002). Overexpression of MT1-MMP by glioma and fibrosarcoma cells causes proteolytic degradation of tTG at the leading edge of motile cancer cells, leading to specific suppression of cell migration and adhesion on fibronectin (Belkin *et al.*, 2001). 80 kDa tTG degradation is mediated *in vitro* by MT1-MMP, MT2-MMP, and MT3-MMP, but not MT4-MMP, and produces fragments of ~53 kDa, ~41 kDa and ~32 kDa. MT1-MMP cleavage at Arg458-Ala459 and His461-Leu462 generates the 53 and 32 kDa fragments, while cleavage at Pro375-Val376 splits the protein in half, providing the 41 kDa fragments. Cleavage at any of these three sites abolishes receptor and enzymatic activity by separating the NH_2-terminal fibronectin-binding domain and the COOH-terminal integrin-binding domain, as well as by inactivation of the catalytic domain. tTG proteolysis suppresses cell adhesion and migration on fibronectin. Reciprocally, fibronectin protects its surface receptor, tTG, from MT1-MMP-mediated proteolysis, thereby supporting cell adhesion and mobility. In contrast, cell migration on collagen matrices is stimulated by tTG degradation. This suggests that the composition of the surrounding ECM might control the proteolysis of adhesion molecules colocalized with MT-MMPs on distinct areas of the cell surface of migrating tumor cells (Belkin *et al.*, 2001).

Additional examination shows that MMP-2 supports its activator MT1-MMP in tTG proteolysis. MMP-2 hydrolyzes cell-associated tTG very efficiently and associates predominantly with the catalytic core domain II of tTG. Furthermore, *in silico* simulations show that during the tTG-MMP-2 interaction, the catalytic site of MMP-2 is probably in very close proximity of the MMP cleavage sites. tTG, in turn, preferentially associates with the activation intermediate of MMP-2, which reduces MMP-2 activation and protects tTG against MMP-2 proteolysis (Belkin *et al.*, 2004). Hence, MMP-2 is as important as MT1-MMP in the degradation of cell surface-tTG, and the cooperation of both MMPs explains the

extensive tTG proteolysis at the normal tissue/tumor boundary. Loss of adhesive and enzymatic activities of tTG at the interface between normal and tumor tissue will reduce cell-matrix interactions and inhibit matrix cross-linking, which might cause multiple pathological alterations in host cell adhesion and mobility (Belkin *et al.*, 2004).

1.4.5 34/67 kDa Laminin Receptor (LR)

As mentioned before, the regulation of sequential tumor cell adhesion and anti-adhesion to ECM components is crucial in the complex process of tumor invasion and metastasis. Besides the modulation of integrin binding to vitronectin and type I collagen, and of tTG binding to fibronectin, MMPs also intervene in cell adhesion to laminin by modification of a major laminin binding molecule, the 34/67 kDa laminin receptor (LR, 40S ribosomal protein SA, p40, colon carcinoma laminin-binding protein, NEM/1CHD4, multidrug resistance-associated protein, MGr1-Ag). Overexpression of LR is strongly correlated with metastatic and aggressive tumor cell phenotypes (Berno *et al.*, 2005). Using thyroid hormone-dependent *Xenopus Laevis* metamorphosis as a model, the 37 kDa LR precursor was identified as a potential physiological substrate of *Xenopus* stromelysin-3 or *X*MMP-11 (Amano *et al.*, 2005b). The highly conserved 37 kDa protein is the precursor of the receptor but the exact manner by which it configures its mature 67 kDa form is not clear. It was suggested that acylation followed by homo- or heterodimerization of the 37 kDa precursor forms the mature 67 kDa laminin receptor. The heterodimer is likely to be stabilized by strong intramolecular hydrophobic interactions between fatty acids bound to the 37 kDa precursor and to an unknown galectin-3 cross-reacting molecule (Buto *et al.*, 1998). The COOH-terminal two-thirds of the LR is located extracellularly and contains a six-amino-acid laminin-binding sequence, whereas the NH_2-terminal third faces the cytoplasm preceded by a short transmembrane domain (see Figure 2). *In vitro* incubation of *X*pro-LR with other (human) MMPs showed that all tested MMPs cleaved LR, with MMP-2 being most efficient, MT1-MMP least efficient, and MMP-3 and MMP-9 cleaving with intermediate efficiencies. However, the cleavage products generated by these MMPs were distinct from those produced by MMP-11. Whereas the cleavage sites of MMP-11, Ala115-Phe116, and Pro133-Ile134, were located between the transmembrane domain and the laminin-binding sequence, cleavage by all other MMPs occurred COOH-terminally of the laminin-binding sequence. As a consequence, only MMP-11 releases LR-fragments that contain the laminin-binding site and may alter cell-laminin interactions. In addition, human LR was cleaved by MMP-11 at the two same sites as in *Xenopus* LR, which means that LR is a conserved substrate for MMP-11 in vertebrates (Amano *et al.*, 2005b). Further investigation using transgenic tadpoles overexpressing MMP-11 showed that LR is cleaved *in vivo* by MMP-11 during intestinal metamorphosis (Amano *et al.*, 2005a). Besides its physiological role in *Xenopus Laevis* development, MMP-11-mediated cleavage of LR is likely to be involved in tumor developement and cancer progression.

MMP-11, similar to LR, is an active partner of invading cancer cells (Rio, 2005). Thus, the coexistence of MMP-11, which is expressed by the fibroblasts within the tumors but not actually by the tumor cells themselves, and LR in tumors may be expected to lead to the cleavage of tumor cell surface LR. This may alter tumor cell-ECM interaction to affect tumor development and cell migration. Peptide G, an LR peptide (residues 161 to 180) containing the laminin-binding sequence (residues 173 to 178) indeed changes the conformation of laminin-1 and increases and stabilizes laminin-1 binding on tumor cells (Magnifico *et al.*, 1996). In addition, peptide G-modified laminin signals tumor cells to change their cytoskeleton to promote motility and invasion. It also induces the expression of a number of proteases characteristic of invasive cancer cells, and leads to increased gelatinolytic activity by MMP-2. Invasiveness of tumor cells conditioned by peptide G-modified laminin was shown to be MMP-2-dependent as it was significantly more inhibited by TIMP-2 than invasiveness induced by native laminin (Berno *et al.*, 2005). Full-length LR shed from malignant cells also induced conformational changes in laminin after binding. In addition, the shed LR modified production of anti-angiogenic angiostatins from plasmin *in vitro*, in this way promoting tumor-associated neoangiogenesis (Moss *et al.*, 2006). As LR-fragments released by MMP-11 contain the laminin-binding site, they might also modulate laminin conformation and enhance tumor cell invasiveness and angiogenesis. Gaining insight into the malignant potential of these soluble LR-fragments may thus be of great interest to elucidate one of the mechanisms that underlies the detrimental effect of MMP-11 in cancer progression.

1.4.6 Syndecan-1, -3, and -4

Syndecans are transmembrane heparan sulfate proteoglycans expressed on all adherent cells (see Figure 2). They are important players in tissue morphogenesis by binding a variety of ECM components such as fibronectin, thrombospondin, various collagens and growth factors via their glycosaminoglycan chains (Beauvais and Rapraeger, 2004). In addition, they can interact with the cytoskeleton through their conserved cytoplasmic domains. Syndecan expression can alter cell adhesion, migration and morphology. The syndecan family is composed of four strongly related proteins (syndecan-1, -2, -3, and -4). The intact ectodomain of each syndecan is constitutively shed from cultured cells as part of normal cell surface heparan sulfate proteoglycan turnover and this process seems to play a role in various pathophysiological events such as host defense, wound healing, arthritis and Alzheimer's disease. How this shedding is regulated remains largely unknown. The ectodomain release of syndecan-1 (CD138) and syndecan-4 (amphiglycan, ryudocan core protein) from NMuMG epithelial and SVEC4–10 endothelial cells is accelerated by various physiological agents activating several intracellular signal transduction pathways. The proteolytic activity responsible for this accelerated shedding is associated with the cell surface and can be specifically inhibited by TIMP-3, pointing to ADAMs as possible mediators (Fitzgerald *et al.*, 2000). However, as will be discussed in a later chapter, secreted MMP-7 also sheds the syndecan-1 ectodomain, releasing a syndecan-1/chemokine KC complex from the mucosal surface of injured lungs, in this way forming a chemokine gradient that directs neutrophils to the site of injury (Li *et al.*, 2002; Shapiro, 2003). HT1080 fibrosarcoma cells also show constitutive shedding of the syndecan-1 ectodomain, but here the shedding activity is inhibited by TIMP-2 and batimastat, but not by TIMP-1. Therefore, the MMP in charge here is not MMP-7, but probably the endogenous MT1-MMP. Recombinant syndecan-1 is cleaved *in vitro* by MT1-MMP as well as by MT3-MMP preferentially at the Gly245-Leu246 peptide bond (Endo *et al.*, 2003). Syndecan-1 expression is associated with inhibition of invasion and reduced migration of HT1080 fibrosarcoma cells. Treatment of these cells with MMP inhibitors increases cell surface syndecan-1 concentrations concomitant with formation of actin stress fibers, which results in further reduction of migration. In contrast, shedding of the syndecan-1 ectodomain by MT1-MMP enhances cell motility on collagen (Endo *et al.*, 2003).

In addition, the shedding of syndecan-4, and to a lesser extent that of syndecan-1, from HeLa cells and human primary macrophages was reported to be accelerated by the chemokine stromal cell-derived factor-1 (SDF-1/CXCL12) and mediated by MMP-9. SDF-1 increases MMP-9 mRNA and MMP-9 activity in HeLa cells, whereas MMP-9 silencing by RNA interference strongly decreases syndecan-1 and syndecan-4 ectodomain shedding accelerated by SDF-1. Shedding of syndecan-4 and syndecan-1 from human primary macrophages is accelerated by SDF-1 as well, and inhibited by anti-MMP-9 antibodies (Brule *et al.*, 2006). As SDF-1 does not bind to soluble syndecan ectodomains, this shedding process by MMP-9 may be part of an autoregulatory/down-regulation cycle: (1) SDF-1 binding to syndecan-4 facilitates its presentation to its receptor CXCR4; (2) SDF-1 activates MMP-9, which removes syndecan-1 and –4 from the cell surface; (3) decreased membrane expression of syndecans down-regulates SDF-1 binding to its receptor (Charnaux *et al.*, 2006). In addition, MMP-9 cleaves off the NH_2-terminal tetrapeptide of SDF-1, which also results in loss of binding to its receptor CXCR4 (McQuibban *et al.*, 2001). As syndecan-4 promotes cell spreading in a β1-integrin-dependent fashion (Thodeti *et al.*, 2003), the role played by its MMP-9-mediated ectodomain shedding in tumor cell migration and metastasis certainly deserves further investigation.

Syndecan shedding has also been observed in physiological systems. *In vitro* release of the syndecan-3 ectodomain from rat Schwann cells, the myelin-forming cells of the peripheral nervous system, is also mediated by an MMP. These Schwann cells transiently express syndecan-3 during embryonic and early postnatal development. Inhibition of syndecan-3 cleavage by several MMP inhibitors such as batimastat/BB-94 and BB-3103 significantly enhances Schwann cell adhesion to the non-collagenous NH_2-terminal domain of α4(V) collagen, which binds syndecan-3 and mediates heparan sulfate-dependent Schwann cell adhesion. MMP-dependent syndecan-3 shedding was also observed *in vivo* in the peripheral nerve tissue of newborn rats, disappearing on day 10, the end of the myelin-forming process in Schwann cells (Asundi *et al.*, 2003). As a consequence, syndecan ectodomain shedding is cell type-specific and this illustrates the varying physiological roles of these proteoglycans in different tissues. As syndecan ectodomain release has an impact on various

pathophysiological processes, the identification of the proteolytic activity in charge might be of great use in the development of new diagnostic and therapeutic strategies.

1.4.7 CD44

CD44 (phagocytic glycoprotein 1 [PGP-1]), HUTCH-1, ECM receptor-III [ECMR-III], GP90 lymphocyte homing/adhesion receptor, hermes antigen, hyaluronate receptor, heparan sulfate proteoglycan, epican) is a ubiquitous multistructural and multifunctional cell adhesion molecule involved in cell-cell and cell-matrix interactions. This family of glycoproteins consists of many isoforms generated by different use of alternatively spliced exons and extensive glycosylation. The most abundant form is the standard hematopoietic type, CD44H, which does not have any variant insertions (Naor *et al.*, 1997). The ECM adhesion activity of CD44 is located in the NH_2-terminal globular domain that forms an important receptor for hyaluronic acid, an abundant glycosaminoglycan that fills interstitial spaces between different tissues and takes part in embryonic development, healing processes, inflammation and tumor development (Toole, 2004). CD44 also binds other ECM components such as type I collagen, fibronectin, fibrin, laminin, and chondroitin sulfate. CD44 has been shown to take part in many important processes such as lymph node homing, T-cell activation, presentation of chemokines and growth factors to traveling cells, wound healing, angiogenesis, metastasis and apoptosis (Naor *et al.*, 1997). The NH_2-terminal ligand-binding domain is followed by a stem sequence, a transmembrane domain and a cytoplasmic tail. The cytoplasmic domain interacts with the actin cytoskeleton and is important for the localization of CD44 at the ruffling edge of migrating cells. MT1-MMP is co-expressed with CD44 on migrating cells and metastatic tumor cells (Seiki, 2002; Seiki, 2003; Itoh and Seiki, 2004). MT1-MMP binds the extracellular portion of CD44H by its hemopexin domain. Hence, CD44H has a major role in ECM degradation, as it forms the connection between MT1-MMP and the actin cytoskeleton, and anchors MT1-MMP at the migrating front (Figure 9) (Mori *et al.*, 2002).

MT1-MMP, in turn, acts as a processing enzyme for CD44H, which is critical for the stimulation of cell motility, probably because it allows the cells to detach from the ECM (Kajita *et al.*, 2001). Co-expression of MT1-MMP and CD44H increases shedding of the commonly produced 65 to 70 kDa fragments of CD44 and generates two additional smaller fragments. Shedding of the 65 to 70 kDa fragments occurs constitutively and is inhibited by TIMP-3, but not by TIMP-1 or TIMP-2, suggesting the proteolytic activity is an ADAM-like protease, although this shedding is increased by MT1-MMP expression. In contrast, release of the two smaller fragments is abolished by TIMP-2 and TIMP-3, but not by TIMP-1, which is the inhibition pattern of MT1-MMP. The cleavage sites (CS) corresponding to the three major fragments are Ser249-Gln250 (CS3) for the big fragment, and Gly192-Tyr193 (CS1) and Gly233-Ser234 (CS2) for the two smaller fragments (Figure 9). These cleavage sites are also detected *in vivo*. In normal tissues, more clipping of the fragment at CS3 occurs than of those of the other sites, whereas in carcinomas CS1 fragments are significantly increased. Thus, CD44 shedding at CS1 and CS3 represents the normal physiological process, whereas increased shedding at CS1 is associated with malignant tumors (Nakamura *et al.*, 2004).

Whereas MT1-MMPs displays the most potent CD44 H shedding activity, other MT-MMPs such as MT2-, MT3-, and MT5-MMP, but not MT4- and MT6-MMP, can also cleave CD44H *in vitro* at CS2, while cleavage at CS1 was hardly detectable (Suenaga *et al.*, 2005). The interaction between the hemopexin domain and CD44 H is conserved in each MT-MMP, which suggests that CD44 is more than a receptor for ECM molecules and may also form a platform for the assembly of various MMPs with their substrates, to modulate cell migration (Figure 9) (Seiki, 2002; Suenaga *et al.*, 2005). Like MT1-MMP, MMP-9 binds directly to CD44 (Yu and Stamenkovic, 1999) and activates latent TGF-β, which contributes to tumor-induced angiogenesis (Yu and Stamenkovic, 2000). In contrast, MMP-7 attaches indirectly to the heparan sulfate chains of the CD44 isoform with a variant exon 3, which also binds substrates of MMP-7, namely mHB-EGF and osteopontin (Yu *et al.*, 2002; Seiki, 2002).

Furthermore, proteolysis of CD44 results in signal transduction to the nucleus. Ectodomain shedding of CD44 induces cleavage in the cytoplasmic portion, which releases the intracellular domain, CD44ICD, into the cytoplasm (Figure 9). CD44ICD migrates to the nucleus where it activates transcription mediated through the 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-responsive element (TRE). One of the potential targets for transcriptional activation by CD44ICD is

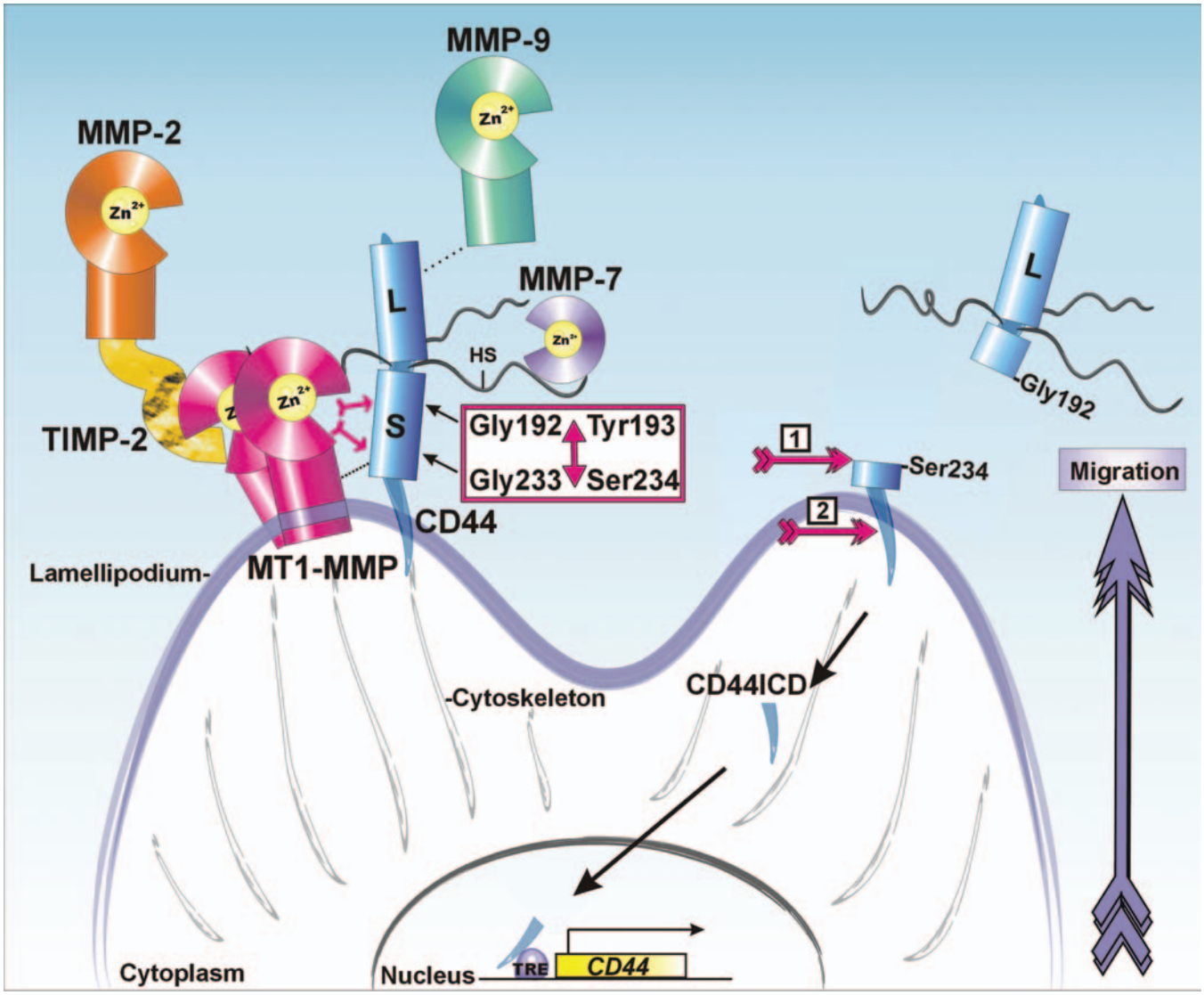

FIGURE 9 **CD44 forms a platform for the assembly of various MMPs with their substrates, to modulate cell migration. The extracellular portion of CD44 binds to the MT1-MMP hemopexin domain (*thin line*), while the CD44 cytoplasmic domain interacts with the actin cytoskeleton, in this way anchoring MT1-MMP on the ruffling edge of migrating tumor cells. MT1-MMP, in turn, acts as a processing enzyme for CD44, which is critical for the stimulation of cell motility, probably because it allows the cells to detach from the ECM. Co-expression of MT1-MMP and CD44 generates two soluble fragments, resulting from proteolysis at Gly192-Tyr193 (CS1) (shown in the figure) and Gly233-Ser234 (CS2) (not shown). Furthermore, ectodomain shedding of CD44 by MT1-MMP (1) induces cleavage in the cytoplasmic portion (2), which releases the intracellular domain, CD44ICD, into the cytoplasm. CD44ICD migrates to the nucleus where it activates transcription mediated through the 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-responsive element (TRE). One of the genes under control of TRE is the *CD44* gene itself, suggesting that CD44 ectodomain cleavage promotes the rapid turnover of CD44 that is required for efficient cell migration. In addition, CD44 acts as a platform to assemble various MMPs with their substrates, clustering proteolytic events that regulate cell migration. MMP-9 also interacts directly with CD44 (*dotted line*), whereas MMP-7 attaches indirectly to the heparan sulfate chains (HS) of CD44. L, ligand-binding domain; S, stem sequence. Adapted from (Seiki, 2002).**

the *CD44* gene itself, suggesting that CD44 ectodomain cleavage and the subsequent intracellular signaling promote the rapid turnover of CD44 that is required for efficient cell migration (Okamoto *et al.*, 2001). As a consequence, disruption of the interaction between MT-MMPs and CD44 might be another strategy to inhibit metastasis of malignant tumors (Peterson *et al.*, 2000; Ueda *et al.*, 2003).

1.4.8 Extracellular Matrix Metalloproteinase Inducer (EMMPRIN)

A search for MMP inducing factors in tumor cells led to the identification of EMMPRIN (Extracellular matrix metalloproteinase inducer) with the numerous synonyms: basigin, leukocyte activation antigen M6 (M6), tumor cell-derived collagenase stimulatory factor (TCSF), neurothelin, OK blood group antigen, OX-47,

gp42, CE9, 5A11, 5F7, HT7, and CD147. EMMPRIN is composed of two Ig domains in the extracellular region, a single transmembrane domain and a short cytoplasmic domain containing 39 amino acids (see Figure 2). It is a highly glycosylated cell surface protein, with the different glycosylation patterns of the 28 kDa native protein accounting for a variable molecular weight, ranging between 44 and 66 kDa. Depending on the cell system, EMMPRIN can stimulate production of MMP-1, -2, -3, -9, MT1-MMP, and MT2-MMP, and only glycosylated EMMPRIN is able to induce these MMPs. The NH_2-terminal Ig domain is required for the MMP induction, but also for the formation of homo-oligomers in a *cis*-dependent manner in the plama membrane. The MMP-inducing function of EMMPRIN in part involves the molecule acting as a counter-receptor for itself, also requiring the NH_2-terminal Ig domain, but in this case the interaction is in a *trans* manner (Gabison *et al.*, 2005; Yan *et al.*, 2005).

EMMPRIN can be released from the cell surface in at least two different ways. A significant amount is released via vesicular shedding, whereby EMMPRIN is initially associated with microvesicles which are quickly degraded upon release from the cells to discharge full-length soluble EMMPRIN. The other pathway is MMP-dependent proteolytic shedding (Gabison *et al.*, 2005). In addition to inducing MMPs, EMMPRIN is cleaved and shed by MMPs or other MPs, because this shedding is inhibited by Zn^{2+} chelators (EDTA and 1,10-phenanthroline) and by the broad-spectrum MMP inhibitor GM6001 (Tang *et al.*, 2004; Haug *et al.*, 2004). In addition, MMP-1 and MMP-2 cleave EMMPRIN at the membrane-proximal region *in vitro* (Haug *et al.*, 2004). Besides release of the intact form, a new EMMPRIN fragment of 22 kDa was identified in the culture media of two tumor cell lines (HT1080 and A431), the shedding of which was enhanced by phorbol 12-myristate 13-acetate (PMA) while that of the intact form was not (Egawa *et al.*, 2006). The EMMPRIN sheddase was hypothesized to be of the MT-MMP family, in view of its expression and inhibition profiles. In addition, HT1080 and A431 cells express MT1-MMP and EMMPRIN was co-purified from cell lysates with MT1-MMP. Knockdown of MT1-MMP with siRNA indeed inhibited the shedding substantially, although MT2-MMP may also contribute to the shedding because knockdown of both MT1- and MT2-MMP produced slightly greater inhibition. The COOH-terminal amino acid of the 22 kDa fragment was identified as Asn98, and MT1-MMP also cleaved at this site in an *in vitro* digestion. Although an additional cleavage site, between Pro93 and Met94, was observed after *in vitro* incubation with MT1-MMP, the corresponding fragment was not identified in cell culture media (Egawa *et al.*, 2006). The Asn98-Ile99 cleavage site is located in the linker sequence connecting the two Ig-like domains (see Figure 2), which means that the 22 kDa fragment contains the NH_2-terminal Ig domain that is crucial for MMP induction and homophilic interactions. The purified 22 kDa fragment indeed retained MMP-inducing activity. Thus, the shedding may down-regulate the cellular functions mediated by EMMPRIN, because MT1-MMP cleaves off the essential distal Ig domain. This regulation may be particularly important at the ruffling edge of migrating tumor cells, as both proteins co-localize there (Egawa *et al.*, 2006). At the same time, the released soluble 22 kDa active fragment may act on cells either in the local tumor environment or diffuse away to act on distant cells to further stimulate MMP and EMPRINN expression and augment the migration and invasion potential of tumor cells.

The pathologic consequences of elevated EMMPRIN expression in tumor growth and metastasis were directly demonstrated using EMMPRIN-overexpressing cancer cells. MDA-MB-436 human breast cancer cells are normally slow-growing cells when they are implanted into nude mice. However, after EMMPRIN gene transfection, these cells adopted a more aggressive phenotype, exhibiting both accelerated growth and increased invasiveness, and increased MMP-2 and MMP-9 expression (Zucker *et al.*, 2001). In addition to stimulating MMP production, EMMPRIN also binds MMP-1 and retains it at the cell surface, an arrangement that may promote turnover of pericellular collagen, thereby also facilitating migration and metastasis. The role of EMMPRIN in tumor cell invasion was confirmed, as EMMPRIN function-blocking antibodies inhibited invasion through a reconstituted basement membrane. Besides its role in the stimulation of invasion, migration and metastasis, EMMPRIN induces angiogenesis via stimulation of VEGF production and multidrug resistance via upregulation of HER2-signaling and cell survival pathway activities (Gabison *et al.*, 2005; Yan *et al.*, 2005). Targeting the release of soluble EMMPRIN molecules by MMPs, may thus be of great interest to limit the expansion and migration of the tumor by restricting the diffusion of malignant EMMPRIN actions.

Besides the elevated EMMPRIN expression on tumor cells, EMMPRIN was also shown to have a broader tissue distribution, including activated T-cells, differentiated macrophages and epithelia. The presence of EMMPRIN in non-tumoral tissue suggests a role in other physiological and/or pathological situations, such as embryonic development, adult tissue homeostasis, atherosclerosis (see Section 2.1.2), arthritis and ulceration (Gabison *et al.*, 2005).

1.4.9 Low-Density Lipoprotein Receptor-Related Protein (LRP)

The low-density lipoprotein receptor (LDL-R) family consists of several related cargo transporters that also inform the cell of changes in its environment by mediating signaling responses. Low-density lipoprotein receptor-related protein 1 (LRP, α2-macroglobulin receptor (α_2-MR), apolipoprotein E receptor (ApoE-R), CD91) is a membrane-bound receptor which mediates the endocytosis of a wide variety of ligands, including lipoproteins, proteases, proteinase inhibitor complexes, ECM components, bacterial toxins, viruses, intracellular proteins and growth factors. In addition to the four clusters of ligand-binding repeats, LRP consists of a 85 kDa membrane-spanning light β-chain (LRP-85) that is non-covalently associated with a 515 kDa large extracellular α-chain (LRP-515) (Figure 10). The cytoplasmic domain, containing 100 amino acids, plays a role in signal transduction by interacting with the cytoplasmic scaffold and adaptor proteins (Strickland *et al.*, 2002; Lillis *et al.*, 2005). In view of its multiple interactions at the cell surface, LRP is a strategic relay in the control of cell behavior. Indeed, LRP not only mediates the endocytic clearance of several major contributors of cancer development, such as ECM components (fibronectin, and thrombospondin-1 and -2) and various proteolytic enzymes (tissue-type plasminogen activator [t-PA], u-PA, MMP-2, MMP-9, and MMP-13), but it is also involved in cell signalling that regulates cell migration and possibly, survival and proliferation. Its own regulation thus appears to be a crucial process, as suggested by two opposite physiopathological examples, with, respectively, enhanced and suppressed LRP expression: the cycling human endometrium (see Section 5.1) and cancer (Emonard *et al.*, 2005).

Invasive cancer cells derived from human prostate or breast tumors express lower levels of LRP, as compared to their non-invasive counterparts. Similarly, LRP expression decreases in late stages of melanocytic tumour progression and in invasive endometrial carcinoma (Emonard *et al.*, 2005). In addition, MT1-MMP, which is highly expressed by the most invasive tumor cells (Seiki, 2003; Sato *et al.*, 2005), has been shown to efficiently degrade LRP (Rozanov *et al.*, 2004a). LRP-515 associates with the MT1-MMP catalytic domain and is highly susceptible to MT1-MMP proteolysis *in vitro* (Figure 10). In cells co-expressing LRP and MT1-MMP, the levels of cellular LRP are decreased and the NH_2-terminal ligand-binding portion is released in the extracellular milieu, thereby destroying the functional activity of the receptor. Similar to MT1-MMP, MT2-MMP, MT3-MMP, and MT4-MMP also degrade LRP (Rozanov *et al.*, 2004a), suggesting that LRP is likely to be susceptible to proteolysis by several individual MT-MMPs in many cancer cell types. LRP is directly involved in the capture, internalization, and clearance of MMP-2, MMP-9, and MMP-13 from the extracellular milieu, and in the translocation into the cell compartment for subsequent lysosomal degradation (Emonard *et al.*, 2005; Van den Steen *et al.*, 2006). In malignant cells, MT1-MMP activates MMP-2, and protects the active enzyme from uptake and clearance by cleaving LRP (Figure 10). Thus, LRP proteolysis by MT1-MMP contributes to maintaining high levels of proteinases such as MMP-2, MMP-9, u-PA, and t-PA in the extracellular milieu, allowing for extensive degradation of the ECM by aggressive migrating cells. In this context, inhibition of MT1-MMP would have a dual beneficial effect in countering tumor invasion and metastasis; first by reducing the activation of pro-MMP-2 and second by enhancing LRP-mediated clearance of ECM-degrading proteases.

1.4.10 MT1-MMP

MT1-MMP expression levels are closely associated with invasiveness and malignancy of tumors, suggesting that MT1-MMP is one of the critical factors for tumor invasion and metastasis. Besides degrading multiple ECM molecules, MT1-MMP contributes to the process of tumor cell metastasis by cell surface proteolysis of various biologically important molecules such as MUC1, tTG, integrins, syndecan-1, CD44, and LRP, as discussed before. Furthermore, it activates pro-MMP-2 and concentrates its proteolytic activity on the cell surface, which is an important step for cancer cells to invade into basal lamina (Sato *et al.*, 2005; Itoh and Seiki, 2006). MT1-MMP is produced as an inactive ~60 kDa zymogen that is activated by furin-like convertases, which cleave at

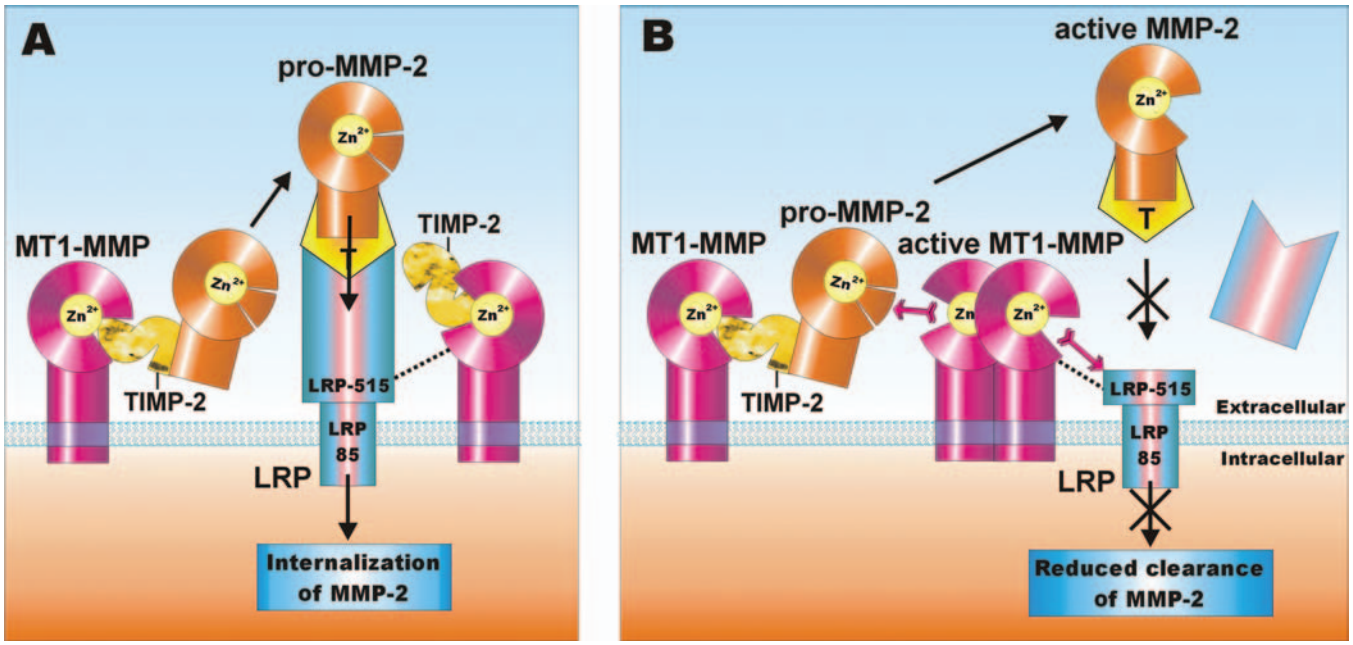

FIGURE 10 **Degradation of LRP by MT1-MMP leads to reduced internalization of MMP-2. (A) When MT1-MMP activity is inhibited by TIMP-2, no MMP-2 activation occurs. MMP-2 binds thrombospondin (T) and is cleared from the extracellular milieu after binding the scavenger receptor LRP. (B) In malignant cells, which often over-express both MT1-MMP and pro-MMP-2, MT1-MMP activates MMP-2. In addition, the 515 kDa large extracellular α-chain of LRP (LRP-515), which associates with the MT1-MMP catalytic domain (*dotted line*), is cleaved by MT1-MMP and the NH_2-terminal ligand-binding portion is released in the extracellular milieu, thereby destroying the functional activity of the receptor. Hence, active MMP-2 is protected from uptake and clearance by LRP and accumulates in the extracellular environment, where it assists MT1-MMP in degrading the ECM in front of aggressive migrating cells. LRP-85, the 85 kDa membrane-spanning light β-chain of LRP. Adapted from (Rozanov *et al.*, 2004a).**

the Arg108-Arg-Lys-Arg motif located between the propeptide and the catalytic domain. Active MT1-MMP (~57 kDa), starting at Tyr112, is then transported to the plasma membrane with the catalytic domain facing the extracellular space, where it cleaves pericellular substrates. In addition to being a sheddase, MT1-MMP is regulated by ectodomain shedding itself. Active MT1-MMP undergoes autocatalytic processing at the cell surface, leading to the formation of an inactive 44 kDa fragment and release of the entire catalytic domain (Figure 11). First, MT1-MMP cleaves itself at the Gly284-Gly285 peptide bond in the hinge region, generating the inactive 44 kDa membrane-bound fragment. The second cleavage takes place at the Ala255-Ile256 peptide bond, in the active site of MT1-MMP, near the conserved methionine turn, a structural feature of the catalytic domain of all MMPs. The released 18 kDa soluble fragment has no catalytic activity and does not bind TIMP-2 (Toth *et al.*, 2002). Hence, the autocatalytic cleavage represents a self-regulatory mechanism that evolved to terminate MT1-MMP-dependent proteolysis both at the cell surface and in the extracellular space. The remaining 44 kDa degradation product can even negatively influence enzymatic activity. For example, it was shown to compete with the full-length enzyme for collagen binding, reducing collagenolytic activity, and cellular invasion of a collagen matrix. Additionaly, it was speculated that the 44 kDa product may indirectly reduce pro-MMP-2 activation by reducing the clustering of MT1-MMP induced by collagen (Osenkowski *et al.*, 2004). Inhibition of MT1-MMP activity slows down autocatalytic enzymatic turnover and consequently the mature form of the enzyme (57 kDa) accumulates on the cell surface while the level of the inactive 44 kDa fragment is reduced. In contrast, autocatalytic processing is promoted in the absence of inhibitors or under conditions of MT1-MMP overexpression. This effect of inhibition on autocatalytic processing unveiled a new paradigm in MT1-MMP regulation because it signaled a potential side effect of MT1-MMP inhibition: enhancement rather than inhibition of activity (Osenkowski *et al.*, 2004).

To make the picture even more complex, mature MT1-MMP is also shed from the cell surface via a non-autocatalytic process that results in the release of various soluble forms (Toth *et al.*, 2002), and, as opposed to the autocatalytic processing, it generates active soluble forms. This shedding occurs *in vivo* and

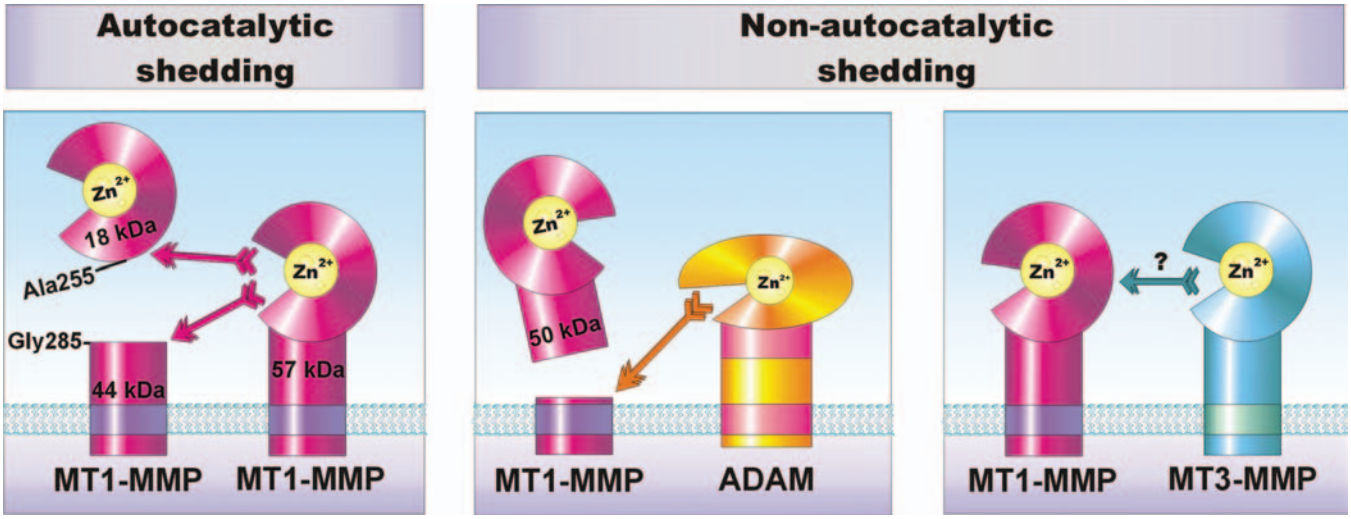

FIGURE 11 **Autocatalytic and non-autocatalytic shedding regulates MT1-MMP activity at the cell surface. Active MT1-MMP undergoes *autocatalytic processing* on the cell surface, leading to the formation of an inactive 44 kDa fragment and release of the entire catalytic domain. First, MT1-MMP cleaves itself at the Gly284-Gly285 peptide bond in the hinge region, generating the inactive 44 kDa membrane-bound fragment. The second cleavage takes place at the Ala255-Ile256 peptide bond, in the active site of MT1-MMP. The resulting 18 kDa soluble fragment has no catalytic activity. MT1-MMP is also shed from the cell surface via a *non-autocatalytic process* that results in the release of active soluble forms. This shedding is mediated by an ADAM and produces a minor form of ~25–32 kDa (not shown) and a major soluble form ~50–52 kDa, wich represents the entire ectodomain, including the catalytic domain. Finally, MT1-MMP may as well be degraded by MT3-MMP. However, no soluble products or cleavage sites of MT3-MMP have been characterized so far. Based on (Osenkowski *et al.*, 2004), (Toth *et al.*, 2006) and (Shofuda *et al.*, 2001).**

produces a minor form of ~25–32 kDa and a major soluble form ~50–52 kDa, wich represents the entire ectodomain, including the catalytic domain (Figure 11) (Toth *et al.*, 2005). The inhibition pattern of this shedding process suggests that it is mediated by an ADAM (Toth *et al.*, 2006).

A third proteolytic mechanism regulates MT1-MMP acitivity at the cell surface. While studying activities of MT1- and MT3-MMP in activated smooth muscle cells, it was shown that when MT3-MMP and MT1-MMP were coexpressed, MT1-MMP degradation was enhanced. This result supports the possibility that MT3-MMP can degrade MT1-MMP (Figure 11), providing another negative regulatory mechanism for MT1-MMP activity in cells such as smooth muscle cells and gliomas, or in tissues such as injured blood vessels and brain, where both MT-MMPs are coexpressed (Shofuda *et al.*, 2001).

Emerging evidence points to internalization as another means of controlling MT1-MMP activity at the cell surface. Classical endocytosis depends on clathrin-coated pits and involves an intracellular pathway in which lysosomes fuse with internalized vesicles, degrading their contents (Shin and Abraham, 2001). MT1-MMP is cleared from the cell surface by dynamin-dependent endocytosis in clathrin-coated pits through its cytoplasmic domain (Jiang *et al.*, 2001). This type of endocytosis was shown essential for MT1-MMP to stimulate cell migration and invasion into Matrigel (Uekita *et al.*, 2001). In addition, MT1-MMP was detected in caveolae (Annabi *et al.*, 2001; Puyraimond *et al.*, 2001). Remacle and coworkers (2003) showed that MT1-MMP is also internalized by a clathrin-independent and caveolae-dependent pathway in HT1080 cells. Caveolar traffic is required for proper MT1-MMP localization, activity and function in migrating endothelial cells (Galvez *et al.*, 2004). Interestingly, internalized MT1-MMP can be recycled to the cell surface, which could represent a rapid mechanism for relocalizing active MT1-MMP at the leading edge during cell migration (Remacle *et al.*, 2003).

Regarding the major role of MTl-MMP in tumor invasion and metastasis (Sato *et al.*, 2005; Itoh and Seiki, 2006), inhibition of its activity is an obvious therapeutic approach to block spreading of the cancer. However, further insight in the proteolytic mechanisms at the cell surface that modulate MT1-MMP activity is a prerequisite in the development of new inhibitors, as unexpected effects such as the paradoxical enhancement of MT1-MMP activity by inhibition of autocatalytic processing, may fatally alter the outcome of inhibitor therapy.

1.4.11 Protease-Activated Receptor-1 (PAR1)

Thus far, the protease-activated receptor (PAR) family comprises four members defined as PAR1 to PAR4 (Coughlin, 2000). They form a unique class of GPCRs that are characterized by a distinctive mechanism of activation. Proteolytic cleavage at specific sites in the extracellular NH_2-terminus exposes a new NH_2-terminus, which serves as a tethered ligand and binds to the second extracellular loop of the same receptor, activating it intramolecularly (Figure 12). The activated PARs initiate signal transduction across the membrane to activate intracellular G proteins that regulate pathways for cell shape changes, secretion, cell proliferation, migration, and adhesion in numerous cell types. Many serine proteases, including thrombin, factor Xa, granzyme A, cathepsin G, elastase, trypsin and plasmin cleave the PAR1 scissile bond at Arg41-Ser42. PAR1 (thrombin receptor, coagulation factor II receptor) is expressed by a wide range of tumor cells and has been shown to be upregulated in breast carcinomas and pulmonary tumors. In addition, the level of expression of PAR1 on tumor cells directly correlates with metastatic potential in both primary breast carcinoma and in established cancer cell lines (Ossovskaya and Bunnett, 2004). Neither thrombin nor other serine proteases appear to be involved in PAR1-dependent breast cancer cell motility. However, MMP inhibitors (1,10-phenanthroline and MMP-200, a hydroxamate inhibitor) block both migration and invasion of breast cancer cells *in vitro*. Only MMP-1, not MMP-2, -3, -7, or -9, cleaves and activates PAR1 upon addition to PAR1-transfected breast cancer cells. MMP-1 inhibitors reduce both cell migration *in vitro* and tumor growth in nude mice (Boire *et al.*, 2005; Pei, 2005). In addition, antagonism of either MMP-1 or PAR1 significantly attenuates tumor-induced endothelial cell activation (ECA), the transformation of the intravascular milieu to a prothrombotic, proinflammatory, and cell-adhesive state, as a result of tumor-endothelial cross-talk (Goerge *et al.*, 2006). Thus, the MMP-1/PAR1 axis functions in both ways: host-derived MMP-1 activating tumor-expressed PAR1, as well as tumor-derived MMP-1 acting on endothelial PAR1 (see Figure 2). Since both MMPs and PARs have also been shown to play important roles in cardiovascular and inflammatory diseases (Ossovskaya and Bunnett, 2004), targetting the MMP-1/PAR1 pathway with therapeutics that block MMP-1 may become an attractive approach in the treatment of a variety of invasive, proliferative, and inflammatory conditions.

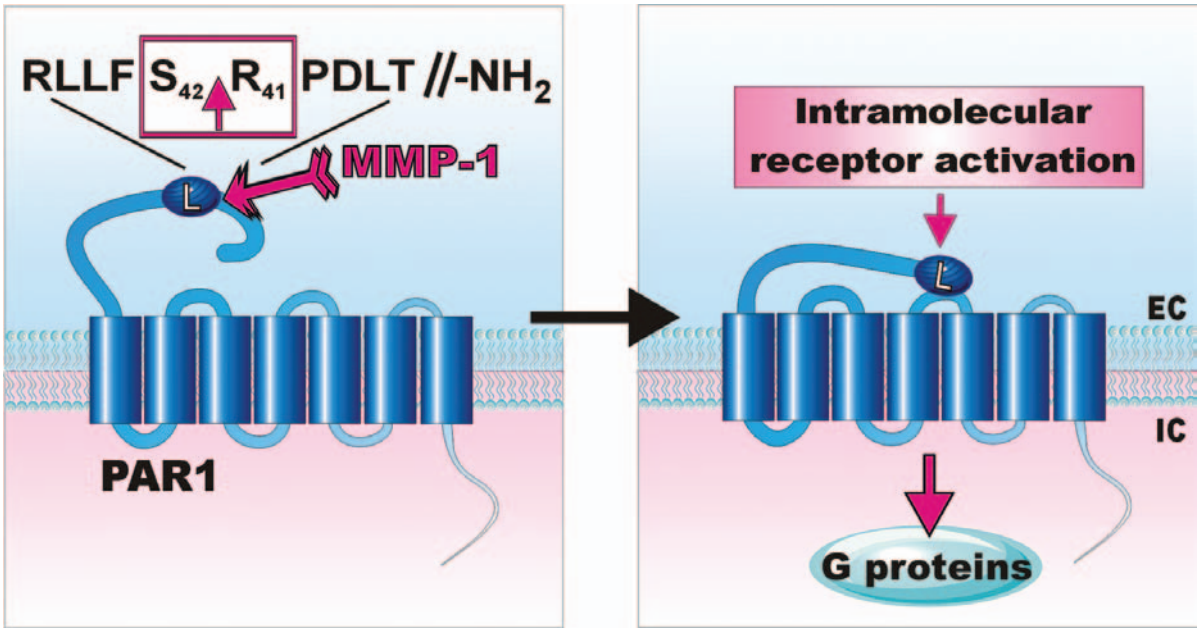

FIGURE 12 **Proteolytic cleavage by MMP-1 in the NH_2-terminus of PAR1 results in intramolecular receptor activation. Proteolytic cleavage by MMP-1 at Arg41-Ser42 in the NH_2-terminus of PAR1 exposes a new NH_2-terminus, which serves as a tethered ligand (L) and binds to the second extracellular loop of the receptor, activating it intramolecularly. Activated PAR1 initiates signal transduction across the membrane to activate intracellular G proteins that regulate pathways for cell morphology, secretion, cell proliferation, migration and adhesion in numerous cell types. EC, extracellular; IC, intracellular. Adapted from Pei (2005).**

1.4.12 Receptor Activator of Nuclear Factor κB Ligand (RANKL)

Prostate cancer deaths are primarily due to metastases that are resistant to conventional therapies. The most common site for metastasis of prostate cancer is the bone, with patients often experiencing severe bone pain, pathological fractures, leukoerythroblastic anaemia, bone deformity, hypercalcaemia, nerve-compression syndromes, and immobility (Mundy, 2002). Osteolytic lesions are produced by the interaction between tumor and bone stroma, commonly referred to as the 'vicious cycle,' whereby tumor cells in the bone can secrete factors such as parathyroid hormone related peptide (PTHrP) that stimulate osteoblast expression of the **r**eceptor **a**ctivator of **n**uclear factor κB **l**igand (RANKL). By binding to its receptor RANK, RANKL has been shown to be essential in mediating osteoclast activation (Figure 13). The osteoclasts degrade the bone matrix using a powerful array of proteases such as the MMPs and the cathepsins (Mundy, 2002). RANKL, also known as tumor necrosis factor ligand superfamily member 11, CD254, TNF-**r**elated **a**ctivation-i**n**duced **c**ytokine (TRANCE), **o**steo**p**rote**g**erin **l**igand (OPGL) and **o**steoclast **d**ifferentiation **f**actor (ODF), is a member of the TNF family. RANKL is required in osteoclast differentiation and activation; T and B cell maturation; dendritic cell survival; and might also play a role in the cardiovascular system. The interaction with its receptor RANK and its decoy receptor osteoprotegerin (OPG) forms a molecular triad which can modulate the bone system, the immune system, and the cardiovascular system (Theoleyre *et al.*, 2004).

To improve overall patient survival and to identify new therapeutic targets, the molecular mechanisms underlying prostate tumor-induced changes in the bone microenvironment need to be elucidated. Therefore, Lynch and coworkers (2005) developed a murine model of rat prostate cancer in the bone environment that mimics the osteoblastic and osteolytic changes associated with human metastatic prostate cancer. MMP-7 was identified as a proteolytic enzyme whose expression correlates with tumor-induced osteolysis. Interestingly, MMP-7 was expressed by the osteoclasts at the tumor-bone interface, and its secretion resulted in the solubilization of RANKL being presented by the osteoblasts, stromal cells or tumor cells in the bone microenvironment (Figure 13). Cleavage of RANKL by MMP-7 occurs at the residues Met145-Met146 in the stalk region of the protein, releasing an active soluble form of RANKL from the cell surface. MMP-3, but not MMP-2, MMP-9, or MMP-13, was also found to cleave RANKL, although the amount of sRANKL produced was significantly less. sRANKL released by MMP-7 was as active and efficient in osteoclast activation as full-length RANKL. The shedding of RANKL is not without significance, because it eliminates the need for close contact between RANKL-expressing cells such as osteoblasts and tumor cells, and RANK-expressing osteoclast precursor cells (Blavier and Declerck, 2005). MMP-7-mediated release of RANKL was also shown *in vivo*, as sRANKL was clearly detected in the tumor-bone interface lysates from wild-type mice, while little or no sRANKL was detected in MMP-7 deficient animals. In addition, tumor-induced osteolysis was significantly reduced in MMP-7 deficient mice (Lynch *et al.*, 2005). Hence, these results make MMP-7 an attractive therapeutic target for the control of cancer-induced bone metastasis.

Interestingly, RANKL was also reported to be solubilized by MT1-, MT2-, MT3-, and MT5-MMP, which will be discussed in a later section (see Section 3.4.2.2).

1.5 Inflammatory Processes and Immune Escape in Cancer

1.5.1 Intercellular Adhesion Molecule-1 (ICAM-1)

Intercellular adhesion molecule-1 (ICAM-1, major group rhinovirus receptor, CD54) is a transmembrane glycoprotein expressed on multiple cell types including leukocytes, epithelial cells, endothelial cells and fibroblasts. It is involved in multiple transient cellular interactions that regulate infiltration, activation and effector functions of leukocytes. Interaction of ICAM-1 with its physiological ligand lymphocyte function-associated antigen-1 (LFA-1, CD11a/CD18 or $\alpha_L\beta_2$, the prototypic β_2-integrin) (see Section 1.4.3) is crucial for leukocyte arrest on endothelial cells, stabilization of interactions between antigen-presenting cells and T lymphocytes by the so-called immune synapse, and for adhesion of cytotoxic T cells and NK cells to their target cells. Besides LFA-1, ICAM-1 binds other ligands such as Mac-1 (Complement receptor 3, CD11b/CD18 or $\alpha_M\beta_2$ integrin), rhinoviruses and malaria-infected red blood cells (Fiore *et al.*, 2002; Hopkins *et al.*, 2004). ICAM-1 expression on resting vascular endothelial cells

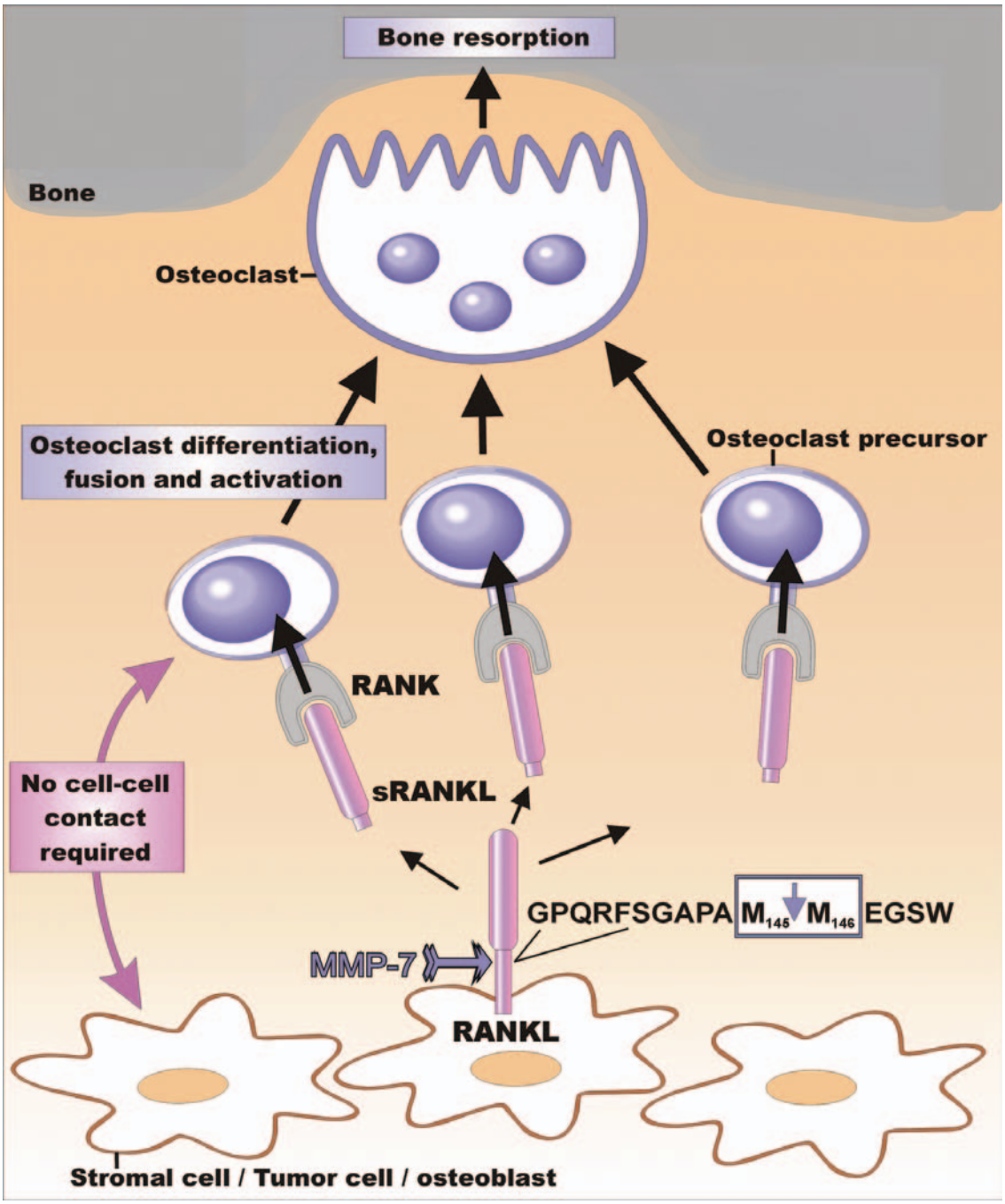

FIGURE 13 **The shedding of RANKL by MMP-7 promotes cancer-induced osteolysis. Osteolytic lesions in bone metastasis are produced by the interaction between tumor and bone stroma, whereby tumor cells in the bone can secrete factors that stimulate osteoblast expression of RANKL. By binding to its receptor RANK, RANKL has been shown to be essential in mediating osteoclast activation. The osteoclasts degrade the bone matrix using a powerful array of proteases such as the MMPs and the cathepsins. MMP-7 has been shown to mediate solubilization of RANKL on osteoblasts, stromal cells or tumor cells in the bone microenvironment. Cleavage of RANKL by MMP-7 occurs at the residues Met145-Met146 in the stalk region of the protein. sRANKL released by MMP-7 was as active and efficient at osteoclast activation as full-length RANKL. The shedding of RANKL is noteworthy because it eliminates the need for cell-cell contact between RANKL-expressing cells such as osteoblasts, stromal cells and tumor cells, and RANK-expressing osteoclast precursor cells. Based on Mundy (2002) and Blavier and Declerck (2005).**

and lymphocytes is low, whereas its expression on monocytes is moderate. Leukemia and carcinoma cells express ICAM-1 as well, and this would make them an easier target for CTLs (Cho *et al.*, 2000). However, ICAM-1 can be released from the surface of tumor cells. This cleavage inhibits cell-mediated cytotoxicity, forming a defence mechanism of primary tumor cells against CTLs and NK cells. The protease in charge of this release

is MMP-9 (Fiore *et al.*, 2002). Sequencing of sICAM-1 peptides points to a cleavage site in the hinge region between Arg441 and the transmembrane domain (see Figure 2), with the Glu442-Val443 peptide bond being the most probable cleavage site (Sultan *et al.*, 2004) in accordance with the substrate specificity of MMP-9 (Kridel *et al.*, 2001). However, the exact site of proteolysis remains to be experimentally determined. In conclusion, MMP-9 might be involved in tumor cell evasion of immune surveillance.

1.5.2 Interleukin-2 Receptor-α Chain (IL-2Rα)

Not infrequently, cancer cell spreading occurs despite the apparent presence of TILs. These autologous CTLs seem unable to display their anti-tumor capacities in the tumor microenvironment. One of the various immune escape mechanisms of cancer cells (see Figure 2) is down-regulation of the interleukin-2 Receptor-α chain (IL2-Rα, p55, TAC antigen, CD25). As IL2-Rα is an essential receptor for the proliferation of T-cells, decreased expression of this receptor *in vivo* may result in poor clonogenicity of TILs and cause immune suppression. An *in vitro* cervical cancer model shows that cervical cancer cells can induce the release of soluble IL2-Rα from encountered T-cells, a process inhibited by TIMPs. Immunohistochemical stainings show abundant expression of MMP-1, MMP-2 and MMP-9 in cervical cancer tissues, and MMP-9, and to a lesser extent MMP-2, are capable of IL-2Rα truncation *in vitro* (Sheu *et al.*, 2001). Consequently, as MMP-9 may contribute to tumor cell evasion of immune surveillance by ectodomain cleavage of ICAM-1 as well as IL-2Rα, its inhibition may be an attractive perspective in countering this type of immune escape of cancer cells.

2. MODIFICATION OF MEMBRANE PROTEINS IN DISEASES AFFECTING VASCULAR AND EPITHELIAL INTEGRITY

2.1 Shedding of MMP Substrates in Cardiovascular Diseases

2.1.1 HB-EGF

sHB-EGF is a potent chemoattractant and mitogen for vascular smooth muscle cells. Atherogenesis in the arterial wall is characterized by the formation of fibrous lesions and the proliferation of neointimal smooth muscle cells. Smooth muscle cells and macrophages in atherosclerotic plaques have indeed been reported to produce large amounts of HB-EGF. Moreover, sHB-EGF-induced proliferation of smooth muscle cells is a key step in the progressive neointimal thickening seen in the development of transplant arteriosclerosis, a major obstacle to long-term graft survival after clinical organ transplantation (Higashiyama, 2004). Release of sHB-EGF is mediated bij ADAM-12 in the heart and by MMP-7 in large mesenteric arteries as a result of ligand binding to GPCRs (see Figure 4), like some adrenoreceptors and angiotensin receptors (Hao *et al.*, 2004). The role of MMP-7 in large mesenteric arteries is taken over by MMP-2 and MMP-9 in the case of increased luminal pressure in small mesenteric resistance arteries (Lucchesi *et al.*, 2004). As the EGFR transactivation is triggered by agonists typically overexpressed in hypertension, its blockade may have therapeutical potential for simultanously inhibiting pathological vasoconstriction and growth in hypertensive disorders like vascular inflammation, atherosclerosis, left ventricular hypertrophy, and cardiac hypertrophy (Shah and Catt, 2003; Hao *et al.*, 2004; Shah and Catt, 2004b).

2.1.2 EMMPRIN

MMPs seem to play an important role in atherosclerotic plaque growth, neointima formation, and plaque disruption by inducing smooth muscle migration and proliferation (*e.g.*, after release of sHB-EGF, as discussed before), and by enhancing ECM degradation (Rouis, 2005). Deposition of low-density lipoproteins (LDLs) in the vessel wall and their oxidative modification seem to initiate, or at least accelerate, the atherosclerotic process by several mechanisms, including promotion of foam cell formation, chemotactic effects on monocytes, and mitogenic effects on smooth muscle cells. In addition, oxidized LDLs increase the expression of MMPs in endothelial cells, monocyte-derived macrophages, and smooth muscle cells (Haug *et al.*, 2004). EMMPRIN (see Section 1.4.8) regulates MMP release and activity in fibroblasts, endothelial cells, and tumor cells (Gabison *et al.*, 2005; Yan *et al.*, 2005). In addition, EMMPRIN has been shown to be expressed in macrophage-rich atheromas from human coronary arteries (Major *et al.*, 2002) and in cultured human coronary artery smooth muscle cells (HCA-SMCs) (Haug *et al.*, 2004). Oxidized LDLs significantly enhanced the release of soluble EMMPRIN (~50 kDa), as well as the release of MMP-1 and MMP-2 into HCA-SMC

supernatants. Oxidized LDL-induced release of soluble EMMPRIN was parallelled by a decrease in cell-associated EMMPRIN. These effects were antagonized by antioxidants as well as by EDTA and the MMP inhibitor GM6001. In addition, MMP-1 and MMP-2 cleaved off the cytoplasmic and transmembrane domains of EMMPRIN *in vitro*. Purified soluble EMMPRIN significantly enhanced MMP-1 and MMP-2 release by HCA-SMC. Thus, oxidized LDLs might induce an amplification cascade of increased MMP activity, enhanced MMP-dependent shedding of soluble EMMPRIN, and EMMPRIN-induced upregulation of MMP production. This cascade might accelerate ECM degradation in atherosclerotic plaques and thereby promote plaque growth and plaque destabilization (Haug *et al.*, 2004). Thus, inhibiting MMP-mediated production of soluble EMMPRIN-molecules, which may diffuse and act on multiple cells, may be an interesting therapeutic tactic to interupt or prevent the atherosclerotic cascade.

2.2 Degradation of Intercellular Junction Proteins in Inflammation, Stroke, Acute Renal Failure and Ophtalmic Pathologies

Adhesion between vertebrate cells is generally mediated by three types of adhesion junctions: 1) tight junctions, 2) adherens junctions, and 3) desmosomes. Together they constitute the intercellular junctional complex, which has an important role in defining the physiological function of a cell; that is, they define whether and how a cell will be integrated in functional structures, such as organ epithelia or stroma (Cavallaro and Christofori, 2004).

Occludin and claudins were identified as the major integral membrane proteins forming the tight junctions in epithelial and endothelial cell sheets. Tight junctions are sites of cell-cell contacts composed of a number of transmembrane and cytoplasmic proteins, assembled into a complex tethered to the cytoskeleton. Paracellular permeability, or the flow of ions and molecules between cells, is regulated by these tight junctions. Both, occludin and claudins have four transmembrane domains and their NH_2- and COOH-terminal ends are located in the cytoplasm. This conformation generates two extracellular loops that are supposed to provide the intercellular interaction sites (Figure 14). The COOH-terminal domains of occludins and claudins serve as binding sites for a complex set of signaling proteins including zonula occludens (ZO)-1, -2 and -3, kinases and phosphatases. Compared to occludin (65 kDa), however, claudins are smaller (20 to 25 kDa). Different cellular sheets have their own unique set of claudin species, and this compositional heterogeneity explains the

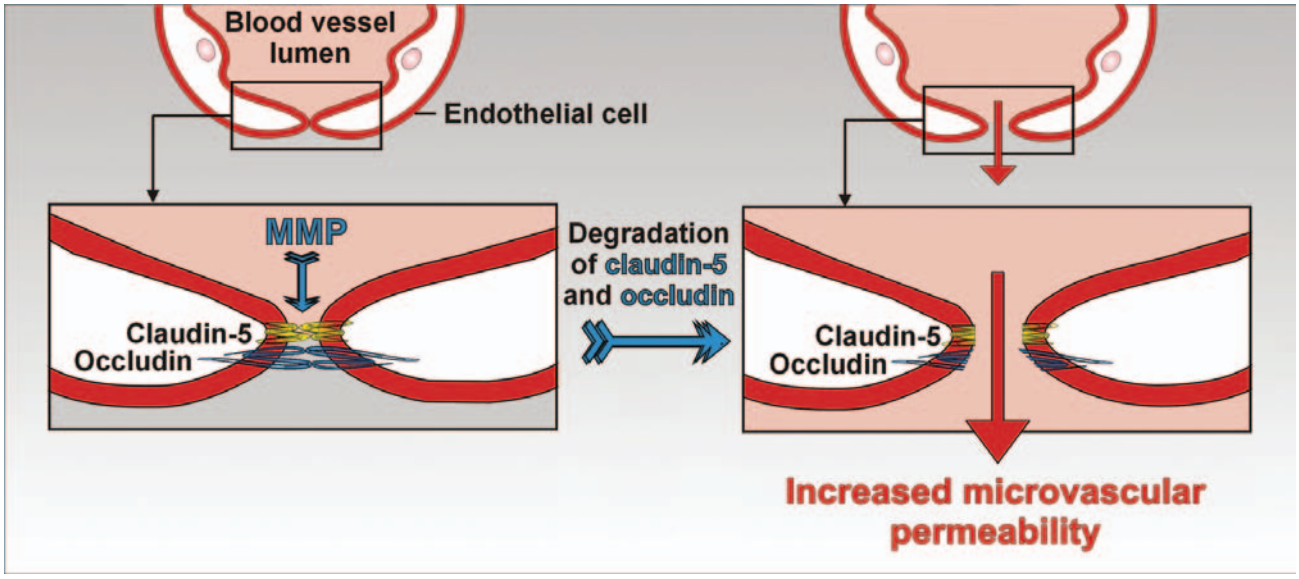

FIGURE 14 **Degradation of tight junction proteins by MMPs disrupts endothelial integrity. Occludin and claudins are the major components of tight junctions in endothelial cell sheets. Both, occludin and claudins have four transmembrane domains and their NH_2- and COOH-terminal ends are located in the cytoplasm. This conformation generates two extracellular loops that are supposed to provide the intercellular interaction sites. Degradation of intercellular junction proteins is mediated by various MMPs and increases microvascular permeability. The widening of interendothelial tight junctions allows for increased solute exchange and immune cell diapedesis, which is a hallmark of inflammation and brain damage after BBB opening.**

diversified barrier properties of tight junctions. Claudin-5 (also named transmembrane protein deleted in velo-cardio-facial syndrome [VCFS]) was shown to be a key molecule in the blood-brain barrier (BBB) in mice (Feldman *et al.*, 2005; Koval, 2006; Bazzoni, 2006; Furuse and Tsukita, 2006). Adherens junctions and desmosomes are cell-cell junctions that are formed by cadherins and additional associated proteins into which actin filaments are inserted.

Degradation of intercellular junction proteins by MMPs disrupts endothelial and/or epithelial integrity and has major consequences in inflammation, stroke, acute renal failure and ophthalmic pathologies, which will be illustrated in the following paragraphs.

2.2.1 Occludin and Claudin-5 Degradation in Inflammation

Increased microvascular permeability is a central hallmark of inflammation and allows for increased solute exchange and extravasation of leukocytes into the inflamed tissue. These inflammatory changes in microvascular permeability are correlated with the reorganization and widening of interendothelial tight junctions. MMPs have been reported to be responsible for the tight junction disruption by degradation of occludin and some claudins (Figure 14) (Alexander and Elrod, 2002). In human umbilical vein endothelial cells and in porcine brain capillary endothelial cells, the tyrosine phophatase inhibitor phenylarsine oxide (PAO) induced increased MMP activity, which was parallelled by severe disruption of cell-cell contacts and degradation of occludin. PAO-induced occludin proteolysis could be prevented by different MMP inhibitors (GM6001 and 1,10-phenantroline) (Wachtel *et al.*, 1999; Lohmann *et al.*, 2004). In addition, MMP-7 has been reported to proteolyse VE-cadherin (see Section 1.3.3). Degradation of intercellular junction proteins by MMPs thus contributes to increased endothelial permeability, a prerequisite for solute exchange and leukocyte extravasation during inflammation.

2.2.2 Occludin and Claudin-5 Degradation in the Blood-Brain Barrier

Tight junction proteins in endothelial cells such as occludin and claudins are major structural components of the BBB formed by components of the neurovascular unit (Bazzoni, 2006). Cerebral ischemia is a complex insult that involves a loss of blood flow accompanied by depletion of oxygen and essential nutrients. *In vitro* models of the BBB have indicated that hypoxia and hypoxia/reoxygenation lead to increased permeability and/or disruption of BBB tight junctions (Hawkins and Davis, 2005). Focal ischemia with reperfusion in spontaneously hypertensive rats lead to opening of the BBB and degradation of occludin and claudin-5 (see Figure 14). Treatment with an MMP inhibitor (BB-1101) prevented the opening and reversed the degradation of the tight junction proteins (Yang *et al.*, 2007). In addition, using green fluorescent protein (GFP)-tagged occludin and live cell imaging, it was shown that monocytes scroll over the brain endothelial surface toward cell-cell contacts, inducing gap formation, which is associated with local disappearance of GFP-occludin, and subsequently traverse the endothelium paracellularly. The broad spectrum MMP inhibitor BB-3103 significantly inhibited endothelial gap formation, occludin loss, and the ability of monocytes to pass the endothelium (Reijerkerk *et al.*, 2006). In addition, after lipopolysaccharide (LPS)-induced opening of the BBB, less BBB disruption was observed in MMP-3 deficient mice than in wild-type controls, as well as diminished degradation of claudin-5 and occludin (Gurney *et al.*, 2006). Hence, use of MMP inhibitors in stroke might reduce or prevent BBB damage by minimizing degradation of tight junction proteins and subsequent immune cell diapedesis.

2.2.3 Degradation of E-Cadherin, N-Cadherin and Occludin in Acute Renal Failure

Acute renal failure (ARF) is characterised by a rapid fall in glomerular filtration rate, clinically manifested as an abrupt and sustained raise in urea and creatinine in plasma. Life threatening consequences include volume overload, hyperkalemia, and metabolic acidosis. ARF is increasingly common and carries a high morbidity and mortality (Hilton, 2006). Although ischemia is a leading cause of ARF, the molecular mechanisms leading to renal injury and failure are not completely understood. In ischemia-induced ARF, a loss of epithelial integrity and shedding of epithelial cells occurs in the tubuli. After injury, both viable and non-viable cells are shed, leaving the basement membrane as the only barrier between filtrate and interstitium, which allows for backleak of the filtrate and tubular obstruction by intraluminal aggregation of cells, proteins, and glycoproteins, such as fibronectin (Bonventre and Weinberg, 2003).

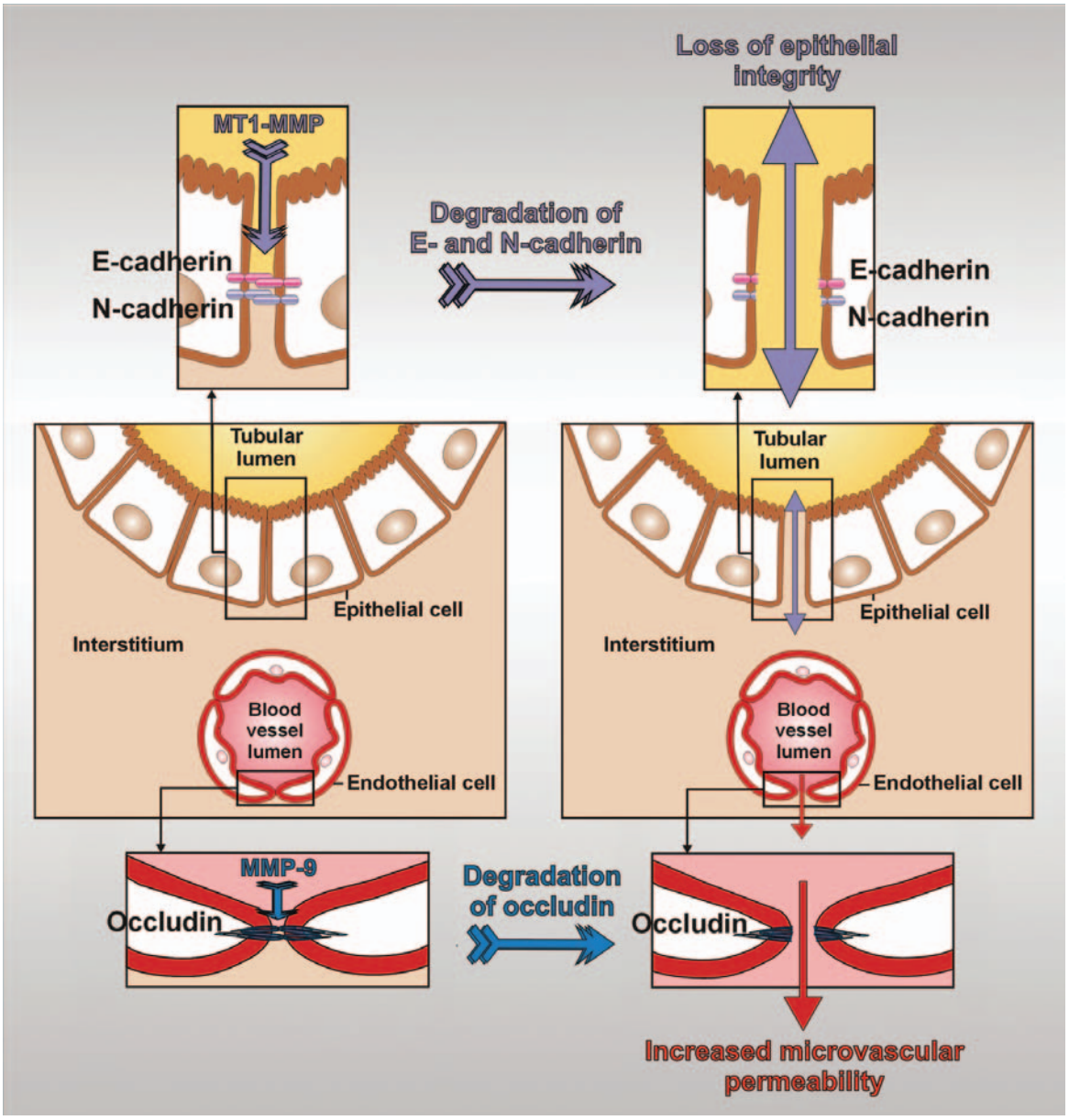

FIGURE 15 **Degradation of intercellular junctions by MMPs in acute renal failure. Cadherins in the adherens junctions and occludins in the tight junctions are key molecules in the regulation of paracellular permeability by the intercellular junction. In ischemia-induced ARF, MT1-MMP-mediated degradation of E-cadherin and N-cadherin results in disruption of epithelial integrity and shedding of epithelial cells in the tubuli, leaving the basement membrane as the only barrier between filtrate and interstitium, which allows for backleak of the filtrate and tubular obstruction by intraluminal aggregation of cells and proteins. In addition, degradation of occludin by MMP-9 leads to the disruption of intercellular endothelial junctions and concomitant cell detachment and vascular injury.**

Application of an *in vitro* model of ischemia-reperfusion resulted in selective fragmentation/loss of E-cadherin and loss of N-cadherin (neural-cadherin, cadherin-2, CD325) levels from normal rat kidney cells that could be blocked by the MMP inhibitors GM6001 and TAPI-0 (Figure 15). TIMP-3 completely blocked both cleavage and/or loss of E-cadherin and N-cadherin, whereas TIMP-2 protected full-length E-cadherin protein expression and TIMP-1 had no effect (Covington *et al.*, 2005). This implies that different MMPs may play a role in E-cadherin and N-cadherin regulation. Chemical inhibitors against a number of soluble MMPs (1, 2, 3, 8, and 9) failed to completely attenuate ischemia-induced E- and N-cadherin loss. Under ischemic conditions, there was increase in active MT1-MMP, and the role of MT1-MMP in ischemia-induced cadherin loss was confirmed by blocking MT1-MMP activity with a neutralizing antibody or by blocking MT1-MMP expression with siRNA constructs, which protected full-length E- and N-cadherin during ischemia and preserved cell-cell

contacts (Covington *et al.*, 2006). Consequently, therapeutic inhibition of MT1-MMP may preserve epithelial integrity and inhibit epithelial cell shedding in ARF. In normal physiology, MMP-7-mediated shedding of E-cadherin is required for the repair of injured lung epithelium (McGuire *et al.*, 2003). Similarly, N-cadherin cleavage also occurs under physiological conditions. Indeed, MT5-MMP was shown to produce a ~35 kDa N-cadherin degradation product in neurons, possibly contributing to mechanisms of synaptic regulation (Monea *et al.*, 2006). Therefore, targetting MT1-MMP in ARF must occur by using extremely specific inhibitors, as not to disturb any vital physiological process.

As discussed above, occludin is one of the major integral membrane proteins forming the tight junctions in endothelial cell sheets. During acute *in vivo* reversible ischemia induced in rat kidneys by vascular clamping, pro-MMP-2, pro-MMP-9, and active MMP-9 were upregulated in the endothelial cell fractions. This increase in MMP-9 during ischemia is accompanied by a lower level of occludin in endothelial fractions. This finding suggests that the induction of MMP-9 during kidney ischemia leads to an increased degradation of occludin (Caron *et al.*, 2005). Thus, ischemia in kidneys could lead to the disruption of intercellular endothelial junctions by MMP-9 and concomitant cell detachment and vascular injury (Figure 15). In addition, active MMP-9 in ischemic kidney may also contribute to vascular basement membrane degradation and increased permeability. Accordingly, blocking MMP-9 and MT1-MMP may be a way to reduce mortality in ARF.

2.2.4 Occludin Proteolysis in Ophtalmic Pathologies

Breakdown of the blood-retinal barrier (BRB) is an early feature of proliferative diabetic retinopathy (PDR) and results in vascular leakage and the developent of retinal edema (Frank, 2004). As in the BBB (see Section 2.2.2), tight junction proteins such as occludin and claudins are an integral structural component of the BRB. Both TGF-β and MMP-9 increased the permeability of retinal endothelial cells and reduced the levels of occludin (see Figure 14) (Behzadian *et al.*, 2001). Treatment of retinal microvessel endothelial cells and retinal pigment epithelial cells with MMP-2 and MMP-9 also revealed specific degradation of occludin, but not of claudin-5 (Giebel *et al.*, 2005). These results suggest that elevated expression of MMPs in the retina may facilitate an increase in vascular permeability by a mechanism involving proteolytic degradation of occludin, followed by disruption of the entire tight junction complex. Thus, a greater understanding of the role of MMPs in altering tight junction proteins in PDR may provide future targets for therapeutic intervention.

Corneal epithelial disease, termed keratoconjunctivitis sicca (KS), is a severe and sight-threatening complication of dry eye syndrome. A key clinical feature of KS is disruption of epithelial barrier function. This results in eye irritation, corneal surface irregularity, blurred and fluctuating vision, and increased risk for corneal ulceration (Pflugfelder, 1998; Pflugfelder *et al.*, 2005). Concentration and activity of MMP-9 in the tear fluid was found to be significantly increased in these eyes, as well as in an experimental murine model of dry eye. Corneal epithelial permeability increased in dry eye wild-type mice, but not in MMP-9 deficient mice. Compared to MMP-9 knockout mice, wild-type mice showed greater desquamification of differentiated apical corneal epithelial cells and this was accompanied by an increase in lower sized (50 kDa) occludin in the corneal epithelia of wild-type mice. The same effects were observed in cultured human corneal epithelial cells treated with active MMP-9 (Pflugfelder *et al.*, 2005). These observations show that increased MMP-9 activity on the ocular surface in response to dryness disrupts corneal epithelial barrier function (Figure 16). The mechanism appears to be similar to BBB and BRB disruption (see above), that is, through disruption of tight junctions by proteolysis of occludin. Accordingly, reducing barrier degradation in human KS by targeting MMP-9 may lessen the severity of clinical disease.

3. SHEDDING OF MMP SUBSTRATES IN THE MODULATION OF INFLAMMATION AND INNATE IMMUNITY

Innate immunity comprises several rapid defence mechanisms against invading microorganisms and other types of damage to the host. During inflammatory reactions, MMP-mediated cleavage of cell surface molecules leads to activation of pro-inflammatory cytokines. This is a fundamental step in the regulation of leukocyte recruitment and homeostasis. In addition, the previously mentioned modifications of mHB-EGF, E-cadherin, integrin subunit precursors, MUC1, ICAM-1 and IL-2Rα, also have major effects on immune responses and wound healing. Finally, proteolysis of cell surface

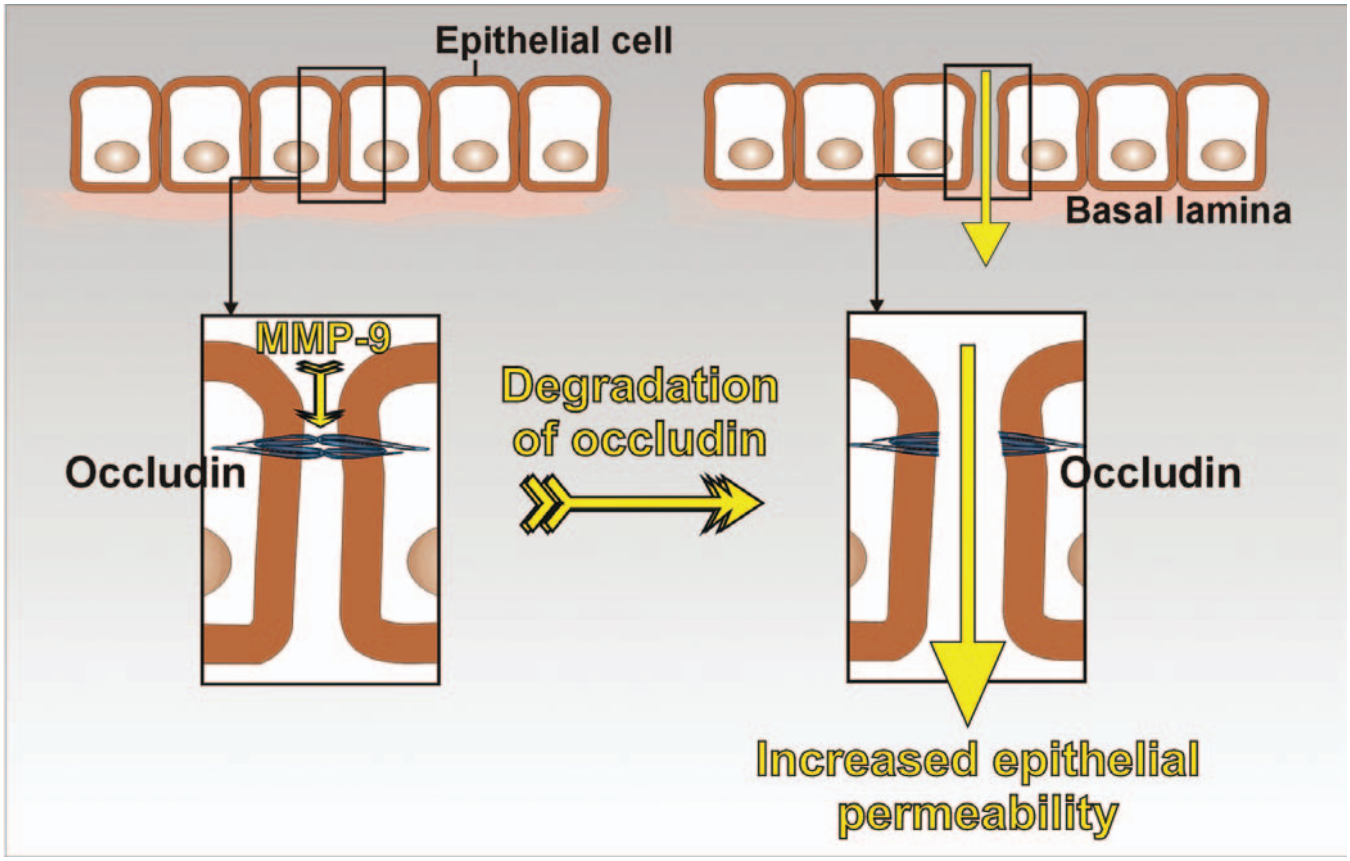

FIGURE 16 **MMP-9-mediated degradation of occludin increases corneal epithelial permeability in keratoconjunctivitis sicca. Occludins in the tight junctions between epithelial cells mediate intercellular adhesion and hence, are essential in epithelial barrier function. Increased MMP-9 activity on the ocular surface in response to dryness leads to degradation of occludin and disruption of the corneal epithelial barrier. Subsequent desquamification of apical corneal epithelial cells leads to keratoconjunctivitis sicca.**

proteins by MMPs plays a non-negligible role in various autoimmune diseases.

3.1 Activation of Membrane-Bound Pro-Inflammatory Cytokines

3.1.1 Tumor Necrosis Factor-α (TNF-α)

Tumor necrosis factor-α (TNF-α, tumor necrosis factor ligand superfamily member 2, cachectin) is a pleiotropic cytokine with potent immunomodulatory and pro-inflammatory properties. Excessive or prolonged production of TNF-α is a feature of septic shock and several important autoimmune diseases like rheumatoid arthritis, Crohn's disease and multiple sclerosis (Kollias *et al.*, 1999). TNF-α is initially expressed on T cells and macrophages as an active 26 kDa membrane-bound protein (pro-TNF-α), which is cleaved by TACE to form the 17 kDa soluble cytokine (Black *et al.*, 1997; Moss *et al.*, 1997; Mohan *et al.*, 2002). TNF-α proteolysis is inhibited *in vitro* and *in vivo* by broad-spectrum metalloproteinase inhibitors (McGeehan *et al.*, 1994; Mohler *et al.*, 1994). MMP-1, MMP-2, MMP-3, MMP-7, and MMP-9 also cleave recombinant pro-TNF-α fusion proteins *in vitro*, as do MMP-12, MT1-MMP, MT2-MMP and MT4-MMP (Gearing *et al.*, 1994; Gearing *et al.*, 1995; Chandler *et al.*, 1996; d'Ortho *et al.*, 1997; English *et al.*, 2000). Of all these MMPs, only MMP-7 processes pro-TNF-α at the natural cleavage site, between Ala76 and Val77 (Table 3). However, this reaction shows a 30-fold lower specificity constant relative to the TACE-mediated proteolysis (Mohan *et al.*, 2002). MMP-7 and MMP-12 are both capable of TNF-α shedding from isolated macrophages. MMP-7-mediated release of TNF-α from peritoneal macrophages is essential for the induction of MMP-3 in coculture with vertebral disc cells. This MMP-3 in turn generates a macrophage chemoattractant, resulting in the macrophage infiltration that is essential for the spontaneous resorption of herniated discs (Haro *et al.*, 2000a; Haro *et al.*, 2000b). TNF-α solubilization by MMP-12 is a crucial step in acute cigarette smoke-induced inflammation, which causes emphysema and chronic obstructive pulmonary disease

(Churg *et al.*, 2003). To summarize, it may be stated that, whereas TACE is the main enzyme responsible for the inducible release of TNF-α in response to bacteria and pathogen-associated molecular patterns (the ligands of Toll-like receptors), MMP-7 and MMP-12 may cause constitutive TNF-α release from macrophages during common processes as tissue resorption and resolution in response to injury (Parks *et al.*, 2004).

3.1.2 Interleukin-1β (IL-1β)

Interleukin-1β (IL-1β, catabolin, endogenous pyrogen, granulocytic pyrogen, leukocytic pyrogen, lymphocyte activating factor, hemopoietin-1, osteoclast activating factor, mononuclear cell factor [MCF]) is a strong pro-inflammatory cytokine that induces fever, inflammation, induction of acute phase reactants, tissue destruction, and, in some cases, shock and death (Dinarello, 2000). IL-1β lacks a typical transmembrane domain and most of its precursor is stored in the cytoplasm. In addition, a small fraction of the precursor can be found in the extracellular space. We discuss its cleavage in the context of the functional analogy with the above mentioned TNF-α proteolysis. IL-1β is primarily produced by activated macrophages, monocytes and polymorphonuclear phagocytes (Delaleu and Bickel, 2004). It lacks a secretory signal peptide and, as a result, it is not secreted through the classical exocytic route, but presumably through exocytosis of secretory lysosomes, a mechanism that still requires further clarification (Andrei *et al.*, 2004; Wewers, 2004). Whereas the COOH-terminal 17 kDa segment has full biological activity (Van Damme *et al.*, 1985), the 33 kDa proform of IL-1β is inactive and its maturation requires proteolytic processing into the mature protein by the IL-1β-converting enzyme (ICE or caspase-1), an intracellular cysteine protease (Kostura *et al.*, 1989). The possibility of extracellular precursor proteolysis and *in vivo* studies with ICE deficient mice suggest the existence of ICE-independent mechanisms of IL-1β activation (Delaleu and Bickel, 2004). Indeed, various proteases cleave recombinant IL-1β *in vitro*, including bacterial enzymes, trypsin, chymotrypsin, leukocyte elastase and granzyme A. However, with the exception of granzyme A, all of these cleavages result in fragments >17 kDa. On the contrary, proteolysis by MMP-2, MMP-3, and especially by MMP-9, yields biologically active forms (Schönbeck *et al.*, 1998). In addition, prolonged incubation of mature IL-1β with MMP-3, and to a lesser extent with MMP-1, MMP-2, and MMP-9, results in degradation of the mature cytokine (Ito *et al.*, 1996). IL-1β is one of the classical inducers of these MMPs in inflammation. In addition, IL-1β is an autoregulating protein with the ability to induce its own gene expression. Thus, at sites of acute or chronic inflammation the presence of MMP-9 might result in biologically active IL-1β, whereas MMP-3 can degrade the active IL-1β form, mediating downregulation of its activities. As IL-1β plays a crucial role in multiple inflammatory and autoimmune diseases, including rheumatoid arthritis, inflammatory bowel disease, and multiple sclerosis (Delaleu and Bickel, 2004), and seems to be involved in tumor invasion and angiogenesis as well (Voronov *et al.*, 2003), it is a principal target for drug development (Braddock and Quinn, 2004). In this regard, understanding IL-1β activity modulation by MMPs is crucial.

3.2 Regulation of Leukocyte Recruitment, Migration and Homeostasis in Inflammation

3.2.1 Syndecan-1

The influx of inflammatory cells to skin or mucosal sites of injury is largely directed by signals from the epithelium, but how these cells generate and modulate chemotactic gradients has not yet been completely elucidated. In murine lungs, a potential mechanism for the formation of neutrophil-attracting chemotactic gradients is based on the interaction of three components of the epithelial tissue: a secreted protease (MMP-7), a cell-bound proteoglycan (syndecan-1) and a CXC chemokine (KC) (Li *et al.*, 2002). When damaged, lung epithelial cells secrete the chemokine KC (and probably other chemokines as well), which binds to preexisting syndecan-1 molecules. MMP-7 is also induced by tissue injury, secreted by wound-edge epithelia and anchored to the heparan sulfate chains of cell surface proteoglycans (Yu and Woessner, 2000). MMP-7 cleaves the syndecan-1 core protein to release the ectodomain-KC complex. The shed complex is then transported, either actively or passively, to the apical surface where it forms a chemotactic gradient which guides neutrophils to the alveolar space (Li *et al.*, 2002). Syndecan-1 ectodomain shedding by MT1-MMP results in enhanced tumor cell migration (see Section 1.4.6), while cleavage by MMP-7 is required for directing neutrophil migration to injured lung tissue. Further clarification of such chemotactic processes is important to allow control of the inflammatory process, in order to improve the removal of

micro-organisms and the repair of tissues, while limiting damage (Li *et al.*, 2002; Shapiro, 2003).

3.2.2 Leukocyte-Selectin (L-Selectin)

Leukocyte-selectin (L-selectin, lymph node homing receptor, leukocyte adhesion molecule-1 [LAM-1], leukocyte surface antigen Leu-8, TQ1, leukocyte-endothelial cell adhesion molecule-1 [LECAM-1], gp90-MEL, MEL-14 antigen, CD62L) is a member of the selectin family of adhesion molecules. L-, P-, and E-selectins each possess a C-type lectin domain, specialized to recognize specific oligosaccharides on mucins and other glycoproteins. Selectins mediate the process of reversible "rolling" in the binding of leukocytes in the bloodstream onto (activated) endothelial cells and specialized endothelial cells lining the high endothelial venules (HEVs) in lymph nodes (Gallatin *et al.*, 1983; Ley and Kansas, 2004).

L-selectin shows the unique property of being cleaved in the membrane-proximal extracellular domain with concomitant release of a soluble fragment that contains the functional lectin and EGF domains. This provides a rapid mechanism for the regulation of L-selectin levels on leukocytes, and hence, controls their ability to migrate into tissues. An extended variety of stimuli, such as chemotactic factors, phorbol ester activation and reagents that cross-link L-selectin, induce L-selectin proteolysis (Preece *et al.*, 1996). The hydroxamic acid-based MMP inhibitor, Ro 31-9790, completely prevents shedding of L-selectin from leukocytes in mice, rats, and humans. *In vitro* reduction of L-selectin-positive leukocytes is mediated by MMP-1, and to a lesser extent by MMP-3, while MMP-2 and MMP-9 have no such effect. However, lymphocytes do not express MMP-1 and MMP-3, and L-selectin levels are not affected by TIMP-1. Moreover, the L-selectin sheddase only acts in *cis* (on the same cell) and not in *trans* configuration (Preece *et al.*, 1996). This shedding is only inhibited by TIMP-3, and not by TIMP-2 (Borland *et al.*, 1999). TACE was identified as the L-selectin sheddase following PMA stimulation (Peschon *et al.*, 1998). However, multiple agents induce L-selectin shedding and have structural requirements that differ from PMA-induced shedding (Smalley and Ley, 2005). In addition, with the use of TACE deficient mice, a significant sheddding of L-selectin was still detected, and a small fraction of this shedding was not inhibited by a metalloprotease inhibitor, suggesting that there may be more than one additional sheddase (Walcheck *et al.*, 2003). Thus, separate proteolytic mechanisms of L-selectin shedding may play a role under a variety of distinctive conditions, for instance to regulate distinct antiadhesive mechanisms.

As L-selectin mediates crucial leukocyte adhesion and migration processes in inflammation, the elucidation of the functional implications of L-selectin shedding was of paramount importance. Using inhibitors of L-selectin shedding it was demonstrated that L-selectin release participates in regulating neutrophil rolling. Moreover, shedding of L-selectin limits leukocyte activation and thus may limit inflammation. Interestingly, plasma of healthy humans and mice contains approximately 1.6 μg/mL of soluble L-selectin. As sL-selectin concentrations of 0.9 μg/mL already reduce lymphocyte migration to peripheral lymphe nodes by over 30%, shedded L-selectin was suggested to regulate normal lymphocyte trafficking and possibly the inflammatory response (Smalley and Ley, 2005). Studies with transgenic mice expressing shedding-resistant L-selectin showed that L-selectin shedding has a role in minimizing reentry of T-cells into peripheral lymph nodes following activation and limits neutrophil adhesion at sites of inflammation. Mice lacking the ability to shed L-selectin on neutrophils also showed reduced neutrophil migration to inflammatory chemokines, suggesting that L-selectin shedding is required for efficient transendothelial migration (Smalley and Ley, 2005). Specification of the other sheddases in charge might contribute to further understanding and control of leukocyte mobility in inflammatory processes.

3.2.3 Kit-Ligand (KitL)

Kit ligand (c-KitL, stem cell factor [SCF], mast cell growth factor [MGF]) is a 31 kDa membrane-bound growth-stimulating cytokine, which is proteolyzed into an active soluble form (sKitL, 164 amino acids) (Heissig *et al.*, 2002). KitL exists in homodimers, and binding to its cell surface receptor c-Kit (SCF receptor or CD117) thus causes dimerization of this TKR. Dimerization of c-Kit in turn results in autophosphorylation and activation of the receptor and of downstream signal transduction proteins, involved in cell proliferation, survival and chemotaxis. KitL is found on stromal cells of the bone marrow, on vascular smooth muscle cells and on endothelial cells, whereas c-Kit is expressed on a variety of cell types, including mast cells, hematopoietic progenitor cells, melanocytes, germ cells and gastrointestinal pacemaker cells (Akin and Metcalfe, 2004).

sKitL plays a key role in the maintenance and reconsitution of the stem and progenitor cell pool. Under steady-state conditions, quiescent c-Kit$^+$ hematopoietic stem cells (HSCs) and circulating endothelial progenitors (CEPs) reside in a niche in close contact with stromal cells from the bone marrow, including osteoblasts. Membrane-bound cytokines such as KitL transmit survival signals and support the adhesion of stem cells to the stroma. Bone marrow ablation (*e.g.*, by cytotoxic agents) induces upregulation of MMP-9, which cleaves KitL. Released sKitL confers signals that enhance mobility of c-Kit$^+$ HSCs and CEPs. As a result, they can translocate into a vascular-enriched proliferative zone, which stimulates differentiation and mobilization into the peripheral blood stream (Heissig *et al.*, 2002). The net number of circulating leukocytes is dependent on the balance between production in the bone marrow, and margination and migration of leukocytes into peripheral tissues and spleen (Opdenakker *et al.*, 1998). Consequently, sKitL release by MMP-9 is crucial for the maintenance of leukocyte homeostasis in the blood, and might be a key step in the recruitment of bone marrow stem cells during cell therapy for cancer and other diseases.

In addition, MMP-9-mediated shedding of KitL also affects smooth muscle function and development of intimal hyperplasia, as this process, characterized by transformation of medial smooth muscle cells from a quiescent contractile to a synthetic proliferative phenotype, seems to be influenced by autocrine sKitL/c-Kit signal transduction (Hollenbeck *et al.*, 2004). The importance of KitL ectodomain proteolysis by MMP-9 is further confirmed by the extended variety of physiological processes affected by KitL/c-Kit signaling, such as maintenance of adult lymphopoiesis in bone marrow and thymus (Waskow *et al.*, 2002), survival, differentiation, chemotaxis, and functional activation of mast cells, melanocyte development and regulation of oogenesis, folliculogenesis, and spermatogenesis (Akin and Metcalfe, 2004).

3.3 Recognition and Clearance of Pathogens in Innate Host Defense

3.3.1 CD14

Surfactant protein-D (SP-D) and CD14 are important innate immune defense molecules that mediate clearance of pathogens and apoptotic cells from the lung. CD14 (monocyte differentiation antigen CD14, myeloid cell-specific, leucine-rich glycoprotein) is a 55 kDa pattern recognition receptor that is present on the surface of monocytes, macrophages and neutrophils. As a GPI-linked receptor, it lacks a cytoplasmic signaling domain and, therefore, it requires interaction with other receptors to elicit its biological responses. CD14 binds LPS and interacts with toll-like receptor 4 (TLR4) and myeloid differentiation protein 2 (MD-2) enhancing MAPK signaling and production of cytokines and chemokines (Antal-Szalmas, 2000). Additional biological functions mediated by CD14 include the transport of lipids, phagocytosis of bacteria, and clearance of apoptotic cells. CD14 also exists as a soluble molecule (sCD14) found in normal human serum and in culture supernatans of monocytes and cell lines. Two molecular forms of sCD14 with different origins have been characterized. First, some CD14 molecules escape GPI anchor attachment. They are stored intracellularly and released spontaneously as 55 to 56 kDa full-length molecules upon a short temperature shift at 37°C. Second, various stimuli such as PMA, interferon (IFN)-γ or LPS, induce shedding of the GPI-anchored CD14, resulting in sCD14 with a molecular mass of 48 to 49 kDa (Antal-Szalmas, 2000). SP-D plays a central role in the pulmonary host defence and is a member of the collectin (collagen-lectin) family, which form multimeric structures consisting of a collagenous NH_2-terminal domain and a globular COOH-terminal carbohydrate recognition domain that binds oligosaccharides at the surfaces of many microorganisms and mediates phagocytosis and killing by phagocytic cells.

SP-D knockout mice (SP-D$^{-/-}$) develop progressive emphysema that is characterized by chronic inflammation, accumulation of surfactant phospholipids, and infiltration with lipid-laden alveolar macrophages (Hartl and Griese, 2006). CD14 is reduced on alveolar macrophages from SP-D$^{-/-}$ mice and is associated with reduced uptake of LPS and decreased production of TNF-α after LPS stimulation. In addition, sCD14 is increased in the bronchoalveolar lavage (BAL) fluid from SP-D$^{-/-}$ mice, while MMP-9 and MMP-12 activities are enhanced in the lungs. Since treatment of macrophages with MMP-1 reduced the level of cell surface CD14 (Bryniarski *et al.*, 2003), MMP-9 and/or MMP-12 were also candidates for the proteolysis of cell surface CD14 to release sCD14 in SP-D$^{-/-}$ mice. sCD14 was indeed significantly reduced in BAL fluid from MMP-9$^{-/-}$/SP-D$^{-/-}$ and MMP-12$^{-/-}$/SP-D$^{-/-}$ mice compared with SP-D$^{-/-}$ mice.

In addition, MMP-12 treatment of RAW 264.7 cells increased sCD14 in the cell culture medium, supporting the concept that MMP-12 cleaves CD14 from the alveolar macrophage cell surface (Senft *et al.*, 2005). Since it was not investigated whether MMP-9 cleaves CD14 *in vitro*, and MMP-12 is decreased in MMP-12$^{-/-}$/SP-D$^{-/-}$ mice, the exact contribution of MMP-9 remains unclear.

In conclusion, SP-D loss results in reduced innate host defence activities, such as decreased LPS uptake and TNF-α production, through shedding of CD14 by MMP-12. In addition, upon recognition of Gram-negative bacterial LPS, sCD14 has a dual regulatory role that is concentration- and environment-dependent. Low concentrations of sCD14 seem to promote beneficial pro-inflammatory responses to LPS at local sites of infection, whereas high circulating sCD14 concentrations may help control potentially harmful systemic responses to LPS (Kitchens and Thompson, 2005). In summary, MMP-12 may be an interesting target in the regulation of microbial clearance and inflammatory processes that are important for host defense and pulmonary homeostasis. However, more investigation will be required to better understand the dual activities of sCD14 in the host during infection.

3.4 Membrane-bound MMP Substrates in Autoimmune Diseases

3.4.1 Bullous Pemphigoid

3.4.1.1 Bullous Pemphigoid Antigen-2 (BP180)

Bullous pemphigoid (BP) is an autoimmune blistering disease of the skin affecting primarily the elderly. Blister formation by detachment of the epidermis from the underlying dermis occurs within the lamina lucida of the basement membrane and is initiated by deposition of IgG autoantibodies and complement components along the basement membrane zone. These autoantibodies are directed against two major hemidesmosomal components, the 230 kDa intracellular protein BP230 (Bullous pemphigoid antigen-1 [BPAG1], Hemidesmosomal plaque protein, Dystonia musculorum protein, Dystonin) and the 180 kDa transmembrane protein BP180 (Bullous pemphigoid antigen-2 [BPAG2]/HD4/Type XVII collagen). Autoantibody deposition causes complement activation and mast cell degranulation, essential for the recruitment and infiltration of inflammatory cells with subsequent degradation of hemidesmosomal and ECM components (Liu, 2003). Proteinases and reactive free radicals from infiltrating inflammatory cells contribute to tissue damage in BP lesions. Blister fluid and fluid at lesional and perilesional regions indeed contain proteolytic enzymes such as neutrophil elastase (NE), plasmin and plasminogen activators, cathepsin G, collagenases and gelatinases, MMP-2 and MMP-9 (Liu *et al.*, 1998; Liu *et al.*, 2000 a; Liu *et al.*, 2000b). *In vitro* experiments show that NE as well as MMP-9 cleave the extracellular, collagenous domain of a recombinant BP180-Glutathion S-transferase (GST) fusion protein. NE deficient and MMP-9 deficient mice are resistant to blister formation after intracutaneous injection of BP180-specific antibodies, although these mice show deposition of autoantibodies at the dermis-epidermis junction. Moreover, blister formation is completely abolished by the α1-proteinase inhibitor (α1-PI), the major NE inhibitor (Liu *et al.*, 2000b) and *in vivo* BP180 is proteolyzed by NE, but not by MMP-9 (Liu *et al.*, 2000a). Consequently, NE is the main tissue-damaging enzyme in murine experimental BP, whereas neutrophil MMP-9 most likely contributes indirectly by inactivating α1-PI, in this way potentiating NE action. In addition, MMP-9 might collaborate with NE in the fragmentation of other proteins, in this way generating and/or maintaining the chemoattractant gradients needed for neutrophil infiltration, an essential step in the blistering process of experimental BP (Liu *et al.*, 1997; Liu *et al.*, 2000b). MMP-9 activation was shown to be plasmin-dependent and independent of MMP-3 (Liu *et al.*, 2005). NE is the BP180-cleaving enzyme in human BP as well (Verraes *et al.*, 2001). In addition, TIMP-1 is present in five-fold molar excess to MMP-9, indicating that cleavage of α1-PI by MMP-9 is less probable in human BP. However, in an *in vitro* model of BP, inhibition of MMP-9 with a specific monoclonal antibody does abolish blister formation (Shimanovich *et al.*, 2004). As a consequence, the contribution of MMP-9 to disease progression is not as clear in humans as it is in mice. This discrepancy might be explained by the differences that exist between murine and human BP. For instance, the majority of biopsies from BP patients show large numbers of eosinophils in their lesional skin, whereas in mice neutrophils are the predominant inflammatory cells. However, some patients may have neutrophil-rich or cell-poor lesions. These varying and different pathological features in human BP indicate that BP is a heterogenous disease. Subepidermal blistering can

be caused by several mechanisms and cell types, the above mentioned immunopathological cascade being one of them (Liu, 2003). Since BP180 is also an autoantibody target in several other subepidermal blistering diseases including cicatricial pemphigoid, herpes gestationis, linear IgA bullous dermatosis, and lichen planus pemphigoides (Liu, 2003), further identification of the tissue damaging-proteases in these skin autoimmune disorders might allow to develop therapies based on the use of synthetic protease inhibitors or inhibitory monoclonal antibodies.

3.4.2 Rheumatoid Arthritis

3.4.2.1 FasL

Rheumatoid arthritis is a chronic inflammatory autoimmune disease, which results in inflammation of the synovial lining and destruction of the adjacent bone and cartilage. Synovial macrophages, fibroblasts and lymphocytes are critical for the pathogenesis of this disease, in which apoptosis may play divergent roles (Liu and Pope, 2003). Insufficient intra-articular apoptosis induces proliferation of the synovial membrane (Sakai *et al.*, 1998; Okamoto *et al.*, 1998). In joints of patients with active rheumatoid arthritis, few apoptotic cells are detected, which might contribute to persistence of the disease (Pope, 2002). MMP-3 concentrations in the synovial fluid of rheumatoid arthritis patients are closely correlated with increased sFasL levels and with disease activity. Consequently, MMP-3 might contribute to the pathogenic mechanism by cleaving FasL, in this way reducing apoptosis (sFasL is a less potent apoptosis-inducer than its membrane-bound precursor, see Section 1.2.1). However, sFasL shedding was not completely blocked by an MMP inhibitor alone, indicating that further investigation is needed for detection of the other proteases involved (Matsuno *et al.*, 2001). Moreover, rheumatoid arthritis is characterized by a very heterogenous disease course with strong synovial proliferation in early disease, whereas in the later stages synovial proliferation is reduced and often replaced by connective tissue (Smith and Walker, 2004). As a consequence, stimulation of apoptosis, for instance by MMP inhibition, might have some therapeutic benefit, but requires further insight into the exact role of apoptosis in the subsequent stages of rheumatoid arthritis.

3.4.2.2 RANKL

Another important TNF family member in rheumatoid arthritis is RANKL. In the absence of RANKL or RANK, osteoclast differentiation from monocyte precursors does not occur (see Figure 13). RANKL is expressed on T-cells and fibroblasts within the synovial inflammatory tissue of patients with rheumatoid arthritis and its expression is regulated by pro-inflammatory cytokines. In animal models of arthritis, blockade of RANKL-RANK interactions, or a genetic absence of RANKL or RANK, protects against joint damage despite the presence of joint inflammation (Schett *et al.*, 2005).

As discussed before, RANKL is a transmembrane glycoprotein that can be converted to a soluble form by ectodomain shedding (see Section 1.4.12). Besides MMP-7, TACE cleaves RANKL *in vitro* (Lum *et al.*, 1999), but further *in vitro* experiments show no difference in sRANKL shedding from fibroblasts with or without TACE (Schlondorff *et al.*, 2001). However, two other RANKL shedding activities can be discerned in these fibroblast cultures. One is induced by the tyrosine phosphatase inhibitor pervanadate and its TIMP inhibition profile is similar to that of several MT-MMPs. The other is constitutive and is insensitive to any TIMP. MT1-MMP overexpression indeed causes increased RANKL release, the ectodomain being cleaved between Met145 and Met146 (Schlondorff *et al.*, 2001). In accordance with these observations, Western Blot of sRANKL shows two bands with molecular weights of 25 kDa and 24 kDa, that are produced by cleavage at Arg138-Phe139 and Met145-Met146, respectively (Hikita *et al.*, 2006). Experiments with inhibitors suggest that the upper band is produced by an ADAM and the lower band by an MMP. A large number of MMPs were shown to cleave RANKL in a RANKL shedding activity screening system, with a fusion protein. However, only MT1-MMP, MT2-MMP, MT3-MMP, and MT5-MMP cleaved full-length RANKL and generated sRANKL with the expected molecular weight. Real-time PCR showed that MT1-MMP mRNA level was much higher than that of MT2-, MT3-, and MT5-MMP in a bone marrow stromal cell line and in primary osteoblasts, suggesting that MT1-MMP is mainly involved in the production of the 24 kDa band. Reduced MT1-MMP expression in primary osteoblasts by siRNA or its deficiency in MT1-MMP knockout mouse osteoblasts reduced RANKL shedding and increased membrane-bound RANKL, which led to increased osteoclastogenic activity in the cells. Conversely, overexpression of MT1-MMP in osteoblasts suppressed osteoclastogenesis. In addition, although sRANKL produced

by MT1-MMP induced osteoclastogenesis from bone marrow macrophages, the culture medium of activated primary osteoblasts did not induce osteoclastogenesis, even when MT1-MMP was overexpressed. These results suggest that membrane-bound RANKL induces osteoclastogenesis more efficiently than sRANKL, and the ectodomain shedding of RANKL by MT1-MMP negatively regulates osteoclastogenesis, which is in accordance with a previous report (Nakashima *et al.*, 2000). Consistent with these *in vitro* observations, soft X-ray images of MT1-MMP deficient mice displayed osteoporosis and a much higher osteoclast number, while the serum level of sRANKL in the MT1-MMP knockout mice was undetectable (Hikita *et al.*, 2006). In conclusion, MT1-MMP was identified as the major endogenous RANKL sheddase in primary osteoblasts and RANKL shedding seemed to downregulate local osteoclastogenesis.

As MMP-7-released sRANKL is as efficient in osteoclast activation as its full-length precursor (see Section 1.4.12 and Figure 13), it seems paradoxical that cleavage by MT1-MMP at the same site in the ectodomain releases a less active form. However, Hikita and colleagues indeed mentioned that the concentration of the sRANKL produced by MT1-MMP in the culture media was more than tenfold lower than the concentration needed to induce osteoclastogenesis with recombinant sRANKL *in vitro*. It is possible that when the expression of RANKL is highly upregulated, MMP-released sRANKL does have substantial effects on general bone metabolism. Hence, further insight into RANKL shedding by MMPs and its concomitant biological consequences *in vivo* may be crucial in the clarification of some osteoarticular pathologies, including rheumatoid arthritis.

3.4.2.3 ADAMTS-4

Aggrecan hydrates the collagen network and thus provides cartilage with its properties of compressibility and elasticity. Degradation of aggrecan can be mediated by two proteases of the ADAMTS family: ADAMTS-4 and ADAMTS-5 (Malfait *et al.*, 2002). ADAMTS-4 (aggrecanase-1, ADMP-1) is synthesized as a protein containing a signal peptide, prodomain, catalytic domain with a Zn^{2+}-binding motif, disintegrin-like domain, thrombospondin Type I motif, a spacer region and a cysteine-rich domain (Tortorella *et al.*, 1999). The intracellular proprotein convertases (PCs), furin, PACE4 and PC5/6 efficiently remove the prodomain through cleavage at Arg212-Phe213, generating an active enzyme. Interestingly, the secreted proteases trypsin, MMP-9, and in a much lesser extent MMP-13, are also effective in removing the prodomain of ADAMTS-4, but the cleavage by MMP-9 occurs at a site other than the PC/trypsin cleavage site. The MMP-9-activated species cleaves the aggrecan peptide substrate, but not native aggrecan, suggesting that the alternative cleavage site changes substrate specificity of the enzyme (Tortorella *et al.*, 2005). In addition to NH_2-terminal activation, it has been suggested that truncation of the COOH-terminus of ADAMTS-4 by a TIMP-1-sensitive GPI-anchored MMP, MT4-MMP, is required for full catalytic activity against aggrecan (Gao *et al.*, 2002). ADAMTS-4 (p100) and MT4-MMP are first processed intracellularly by furin-mediated removal of the prodomains, followed by their association in the secretory pathway. The GPI-anchored MT4-MMP/ADAMTS-4 (p68) complex moves to the cell surface, where MT4-MMP removes the spacer domain of ADAMTS-4 (p68), generating the p53 form, which can be found in association with both chondroitin and heparan sulfate on syndecan-1. ADAMTS-4 (p40) is generated by removal of the spacer region as well as the cysteine-rich domain, and appears in the medium. Analysis with specific antibodies shows that MT4-MMP cleaves at the Lys694-Phe695 and Thr581-Phe582, to generate the p53 and the p40 form, respectively (Gao *et al.*, 2004). Modification of synthesis, furin-mediated activation and/or GPI-anchoring of MT4-MMP may thus be ways to control ADAMTS-4 activation specifically, in this way preventing the destructive aggrecanolysis seen in human joint diseases such as arthritis. Therapeutic agents interfering with the association of ADAMTS-4, MT4-MMP and syndecan-1 on the cell surface might also be promising in this cartilage-protecting approach. In addition, if MMP-9 activates ADAMTS-4 extracellularly, it will be important to establish which ECM proteins are cleaved by MMP-9-activated ADAMTS-4, and whether it has an impact on cartilage turnover in joint diseases.

3.4.3 Multiple Sclerosis

3.4.3.1 Myelin Basic Protein (MBP)

Multiple sclerosis is a chronic neurological disorder of the central nervous system (CNS), characterised by the breakdown of the BBB, perivascular infiltration of inflammatory cells and demyelination. Extracellular

proteases, such as some MMPs, plasmin and plasminogen activators form an amplification cascade in this autoimmune disease by: 1) increasing the permeability of the BBB; 2) demyelination through degradation of myelin basic protein (MBP, myelin A1 protein, myelin membrane encephalitogenic protein), a major component of the myelin sheath and one of the most abundant proteins of the CNS; 3) release of antigenic peptides which contribute to autoimmunity; and 4) facilitating infiltration and migration of immune cells through the ECM and the basal membrane (Opdenakker and Van Damme, 1994; Chandler *et al.*, 1995; Cuzner and Opdenakker, 1999; Opdenakker and Van Damme, 2002; Opdenakker *et al.*, 2003). Various MMPs cause MBP degradation *in vitro*, the most active enzymes on this substrate being MMP-2 and MMP-12, followed by MMP-3; and by MMP-1, MMP-7, and MMP-9 with comparable but lesser activity (Proost *et al.*, 1993; Chandler *et al.*, 1995; Chandler *et al.*, 1996). MMP-9 activity is detected in the cerebrospinal fluid of patients with multiple sclerosis and other neurological inflammatory disorders (Gijbels *et al.*, 1992). Furthermore, young MMP-9 deficient mice are resistant to experimental autoimmune encephalomyelitis (EAE), a murine model for multiple sclerosis (Dubois *et al.*, 1999). MMP-9 generates encephalitogenic peptides by cleavage of human MBP at four different sites *in vitro*: between Phe90-Lys91, Ser110-Leu111, Phe114-Ser115, and Asp133-Tyr134 (Proost *et al.*, 1993). Furthermore, *in vivo* MBP degradation is also exerted by MMP-9, as MBP proteolysis after transient focal ischemia is significantly reduced in MMP-9 knockout mice compared with wild-types (Asahi *et al.*, 2001).

3.4.3.2 NG2 Proteoglycan

Conversely, besides these disease-promoting roles in multiple sclerosis, MMP-9 also has a beneficial role after demyelinating CNS injury. MMP-9 knockout mice are impaired in myelin reformation after lysolecithin-induced demyelination. This might be explained by the role of MMP-9 in the clearance of injury-induced deposits of NG2 proteoglycan (chondroitin sulfate proteoglycan NG2, melanoma-associated chondroitin sulfate proteoglycan), an inhibitory transmembrane proteoglycan that retards the maturation and differentiation of oligodendrocytes needed for remyelination. Consequently, MMP-9 is needed for an efficient remyelination and in this way also has a reparative function in multiple sclerosis (Larsen *et al.*, 2003).

3.4.3.3 β-Dystroglycan

Dystroglycan (dystrophin-associated glycoprotein 1) exists as an extracellular highly glycosylated α-subunit and a non-covalently associated transmembrane β-subunit, which are products derived from one gene and result from posttranslation processing of the glycoprotein. The α-subunit binds to several extracellular ligands, including laminin, agrin, perlecan and neurexin, while β-dystroglycan connects intracellularly to dystrophin, which binds to the actin cytoskeleton. Dystroglycan is expressed in many cell types and the broad range of ECM ligand partners indicates that it has an important role in the assembly and maintenance of basement membranes (Barresi and Campbell, 2006).

The 43 kDa β-subunit was shown to be processed to a 30 kDa fragment by MMPs, the inhibitor profile pointing to MMP-2, MMP-9, and MT1-MMP as possible candidates (Yamada *et al.*, 2001). Kaczmarek and coworkers observed β-dystroglycan breakdown in the brain in response to kainate in a temporal pattern parallel to increased MMP-2 and MMP-9 activities (Kaczmarek *et al.*, 2002). This temporal parallelism of MMP-2 increase and β-dystroglycan proteolysis was also observed in the superior cervical ganglion after postganglionic nerve lesion (Leone *et al.*, 2005; Paggi *et al.*, 2006). However, Agrawal and coworkers (2006) were the first to show that MMP-2 and MMP-9 cleave β-dystroglycan *in vitro* as well as *in vivo*, as they identified β-dystroglycan as a key substrate of MMP-2 and MMP-9 in EAE. This study demonstrates that leukocyte cuffing, as it occurs in human multiple sclerosis, is the consequence of the containment of leukocytes between the endothelial and the adjacent parenchymal basement membranes around the blood vessels in the CNS. Obviously, as long as the leukocytes are contained by the parenchymal basement membrane, synthesized by the astrocyte endfeet of the glia limitans, no disease symptoms occur. Gelatinases, both MMP-2 and MMP-9, cleave *in situ* β-dystroglycan in the parenchymal basement membrane and this process coincides with barrier breakdown, infiltration of the CNS parenchyma by leukocytes and development of disease symptoms. As a result, double MMP-9 and MMP-2 knockout mice are completely resistant against disease development. This cleavage is a critical event, since this MMP substrate is localized on the interface between the extracellular milieu and the astrocytes: its cleavage results in complete desintegration of the glia limitans structure and function (Agrawal *et al.*, 2006).

Proteolysis of β-dystroglycan by MMPs may also contribute to cancer growth and spread (Jing *et al.*, 2004), as well as to skeletal muscle degeneration in the muscular diseases sarcoglycanopathy and Duchenne muscular dystrophy (Matsumura *et al.*, 2005).

Administration of synthetic MMP inhibitors to rodents with EAE significantly ameliorates clinical symptoms and pathological signs (Cuzner and Opdenakker, 1999). Therefore, a major challenge in multiple sclerosis therapy development is the design of selective and specific MMP inhibitors, taking into account the multiple disease-affecting functions of some MMPs, like for instance the role of MMP-9 in demyelination as well as in remyelination (Opdenakker *et al.*, 2003).

3.4.4 Systemic Sclerosis

3.4.4.1 uPAR

Systemic sclerosis (Ssc), also called scleroderma, is a clinically heterogenous, systemic disorder which affects the connective tissue of the skin, internal organs and the walls of blood vessels. It is characterized by alterations of the microvasculature, disturbances of the immune system and massive deposition of collagen and other matrix substances in the connective tissues (Haustein, 2002; Chen *et al.*, 2003). Defective angiogenesis, resulting in tissue ischemia, is particularly prominent in the diffuse form of Ssc. As the u-PA/uPAR system is critical in angiogenesis (see Section 1.3.1), microvascular endothelial cells (MVECs) were isolated from the dermis of healthy individuals and from the dermis of patiens with diffuse Ssc to examine u-PA and uPAR levels. Compared with MVECs from healthy skin, MVECs from Ssc patiens showed higher expression of uPAR. However, in Ssc MVECs, uPAR undergoes truncation between its D1 and D2 domains (see Figure 7), which impairs u-PA binding to uPAR. These properties of Ssc MVECs were associated with poor spontaneous and u-PA-dependent invasion, proliferation, and capillary morphogenesis. The uPAR cleavage occurring in Ssc MVECs was associated with overexpression of MMP-12 and both a general hydroxamate inhibitor of MMP activity and anti-MMP-12 antibodies restored this Ssc MVEC-induced impaired functioning (D'Alessio *et al.*, 2004). In addition, fibroblasts from Ssc patients overexpress MMP-12, which cleaves uPAR of MVECs, thus contributing to the failure of Ssc-endothelial cells to induce an efficient angiogenic programme (Serrati *et al.*, 2006). The overexpression of MMP-12 by both Ssc endothelial cells and Ssc fibroblasts indicates that MMP-12 overproduction may have a critical pathogenic role in Ssc-associated vascular alterations. Hence, selective inhibitors of MMP-12 are likely to be efficient at inducing reversal of Ssc-associated lack of angiogenesis, whereas MMP-12 itself seems disease limiting in cancer by blocking angiogenesis (see Section 1.3.1).

4. PROTEOLYSIS OF CELL SURFACE PROTEINS IN NEURODEGENERATIVE DISORDERS: ALZHEIMER'S DISEASE

Alzheimer's disease is a neurodegenerative disease of the CNS associated with progressive loss of recent memory, resulting in dementia. A clinical diagnosis of Alzheimer's disease is confirmed by observing neuritic (amyloid) plaques and neurofibrillary tangles in the hippocampus, amygdala, and association neocortex (Selkoe, 2004). The plaques are formed extracellularly and are composed of the 42- and 40-residue β-amyloid proteins (Aβs). In healthy individuals, 90% of the Aβs produced by brain cells throughout life are Aβ40 peptides *versus* only 10% of Aβ42. In the cortex of mentally normal elderly patients the Aβ deposits found are almost exclusively 'diffuse' plaques that seem to represent the relatively benign precursor lesions. These diffuse plaques are composed of Aβ42, which is far more prone to aggregation than the slightly shorter and less hydrofobic Aβ40. The Aβ hypothesis predicts that gradual elevation of Aβ42 levels in brain interstitial fluid, and perhaps also inside neurons, can lead to the oligomerization of the peptide and eventually to its fibrillization, that is, amyloid formation. Such insoluble amyloid fibrils are characteristic for 'neuritic' plaques which are associated with local microglial activation, astrocytosis, and cytokine and acute phase protein release. These local inflammatory processes and other neurotoxic effects of oligomerized Aβs finally lead to extensive neuronal and synaptic dysfunction and neurotransmitter deficits, which all contribute to memory impairment (Selkoe, 2004; Walsh and Selkoe, 2004).

4.1 Amyloid Precursor Protein (APP) and β-Amyloid Proteins (Aβs)

Aβ is derived from a membrane-bound Aβ precursor protein (APP, cerebral vascular amyloid peptide (CVAP), protease nexin-II (PN-II), Alzheimer disease amyloid protein, PreA4) after sequential cleavages by

a β-secretase and a γ-secretase (Selkoe, 2004). In normal processing of APP, the most common cut is carried out by the α-secretase and occurs between Lys 687 and Leu 688, that is 12 residues NH_2-terminal to the transmembrane region, between Lys16 and Leu17 of the Aβ region (Figure 17). This cleavage creates a large, soluble ectodomain fragment (sAPPα) that is released from the cell surface and leaves a COOH-terminal fragment of 83 amino acids embedded in the membrane. APP is also cleaved between Met671 and Asp672 by a β-secretase, releasing a slightly shorter fragment of APP (sAPPβ) and leaving a membrane-embedded COOH-terminus of 99 amino acids. This 99 amino acid fragment can then be cleaved by a γ-secretase to create Aβ. In summary, proteolysis by an α-secretase releases sAPP from the cell surface, while sequential cleavages by a β- and a γ-secretase lead to Aβ formation (Selkoe, 2004).

The α-secretase is a membrane-associated metalloproteinase, the activity of which is readily inhibited by hydroxamate-based synthetic inhibitors (Higashi and Miyazaki, 2003b). Furthermore, release of sAPP is extremely diminished in fibroblasts from TACE deficient mice *vs.* control mice, making TACE a prime candidate for the α-secretase activity (Buxbaum *et al.*, 1998). MMP-2 is capable of processing APP *in vitro,* but it is not clear whether the cleavage occurs between Lys 687 and Leu 688, like an α-secretase (Miyazaki *et al.*, 1993; Miyazaki *et al.*, 1994) or between Glu668-Val669,

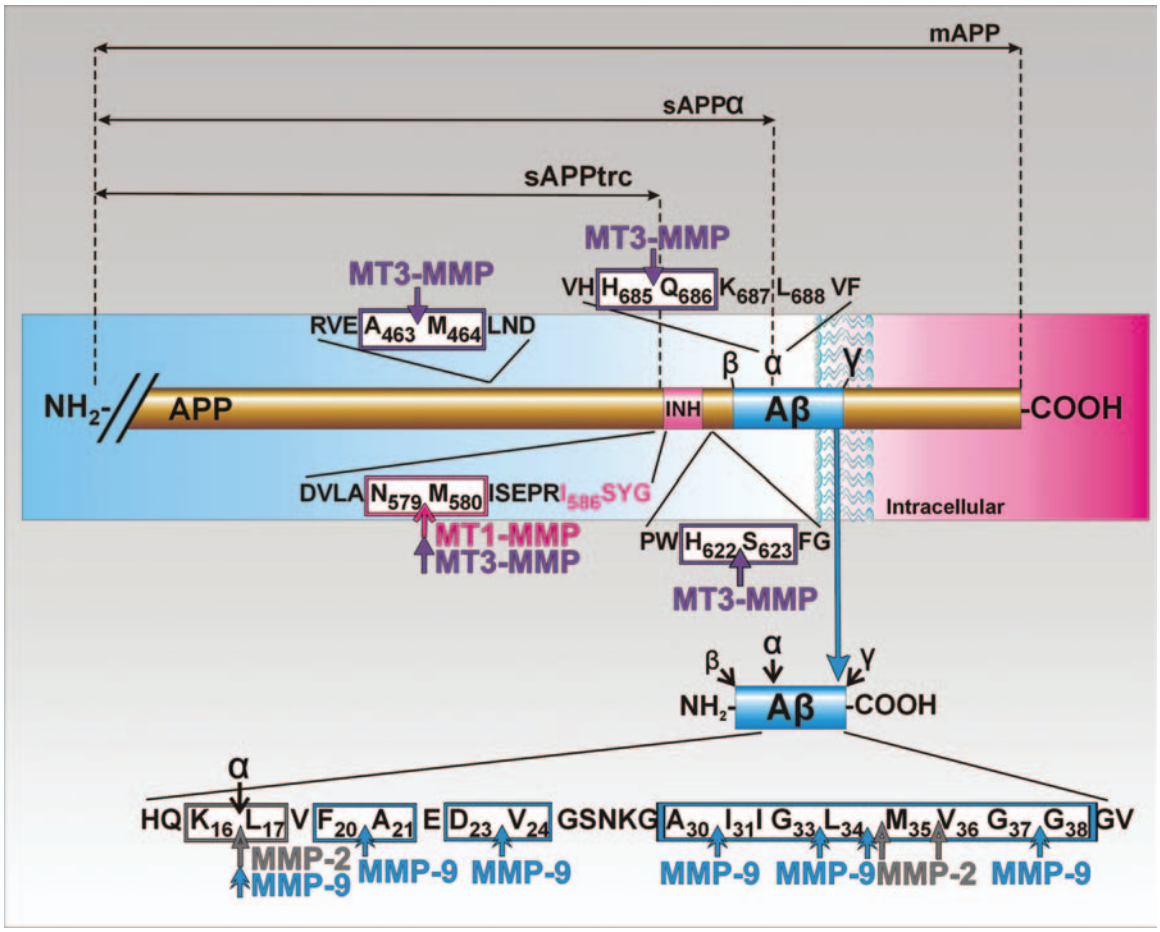

FIGURE 17 **Differential cell surface proteolysis of APP and Aβ leads to accumulation or clearance of pathogenic Aβ peptides in Alzheimer's disease. Aβ (Aβ-amyloid protein) is derived from the membrane-bound Aβ precursor protein (mAPP) after sequential cleavages by a β-secretase and a γ-secretase. However the most common processing of APP is carried out by the α-secretase and occurs between Lys 687 and Leu 688, that is between Lys16 and Leu17 of the Aβ region. This cleavage creates a large, soluble ectodomain fragment (sAPPα). A different processing of APP, between Asn579-Met580, is catalyzed by MT1-MMP and MT3-MMP, and releases a COOH-terminally truncated APP fragment (sAPPtrc) that lacks the MMP-2 inhibitor domain (INH). MT3-MMP cleaves the APP ectodomain at additional sites: at Ala463-Met464, His622-Ser623, and His685-Gln686, which is near the α-secretase cleavage site. Similar to α-secretase cleavage, proteolysis of the His685-Gln686 peptide bond destroys the Aβ-sequence, which diminishes pathologic Aβ formation and accumulation. Once released, Aβ may be cleared by MMP-2 and MMP-9, which cleave it at multiple sites. In addition, MMP-9 has been shown to degrade Aβ fibrils, by proteolytic cleavage at Phe20-Ala21 and Ala30-Ile31. Adapted from (Higashi and Miyazaki, 2003b).**

like a β-secretase (LePage *et al.*, 1995). Furthermore, *in vivo* studies with MMP-2 knockout mice suggest that MMP-2 does not play an essential role in the generation of APP fragments at physiological conditions (Itoh *et al.*, 1997). MMP-3 was also reported to cleave a synthetic APP peptide spanning the β-secretase cleavage site at the residues Glu668-Val669 and Glu674-Phe675 *in vitro*, but this was not further investigated (Rapala-Kozik *et al.*, 1998). APP binds to the ECM and contains an MMP-2-inhibitory domain, which is located in the COOH-terminal glycosylated region of the sAPPs (Miyazaki *et al.*, 1993) within the ISYGNDALMP sequence corresponding to the residues 586 to 595 of APP (Higashi and Miyazaki, 2003a). Another cell-bound MMP might thus be responsible for APP proteolysis. Cell-mediated activation of MMP-2 is indeed accompanied by a different processing of APP, between Asn579 and Met580, which is catalyzed by MT1-MMP and releases a COOH-terminally truncated APP fragment (sAPPtrc) that lacks the MMP-2 inhibitor domain (Figure 17) (Higashi and Miyazaki, 2003b).

An analogy exists in cancer biology, in which APP proteolysis at the ruffling edge of migrating cancer cells (see Figure 2) may be another mechanism by which MT1-MMP promotes migration and invasion (see Section 1.4). It may indeed be suggested that the interplay of MT1-MMP, sAPPtrc, and MMP-2 takes part in the regulation of MMP-2-catalyzed ECM degradation. On the cell surface, where the MT1-MMP concentration is low, APP is liberated mainly as sAPP that inhibits MMP-2 activity. sAPP (as well as APP) binds to the ECM, which might concentrate and enhance the inhibitory effects of sAPP, in this way protecting the ECM near the cell surface from MMP-2 degradation. In contrast, a high (local) concentration of MT1-MMP converts APP to sAPPtrc, which displaces ECM-associated APP or sAPP, thereby removing the MMP-2 inhibitory activity from the ECM. In addition, MT1-MMP activates MMP-2, which can then freely exert its proteolytic activity on the ECM (Higashi and Miyazaki, 2003b). Since autodegradation is a highly critical step in the regulation of MT1-MMP activity (see Section 1.4.10), excess expression of substrates such as APP or syndecan-1 may interfere with the autodegradation and consequently augment the MT1-MMP activation of MMP-2, further promoting MMP-2 proteolytic action.

However, MT1-MMP is not the only MT-MMP expressed in the brain as MT3-MMP expression levels are highest in the brain and localized in microglial cells in all brain tissues (Takino *et al.*, 1995; Yoshiyama *et al.*, 1998), whereas MT5-MMP is also present in all brain tissues, but most strongly expressed in cerebellum (Sekine-Aizawa *et al.*, 2001). After cotransfection, MT3-MMP and MT5-MMP, but not MT2-, MT4- and MT6-MMP, indeed caused cleavage and shedding of the APP ectodomain with a fragmentation pattern almost identical to that of MT1-MMP. MT3-MMP induced shedding of APP most efficiently, followed by MT1-MMP and MT5-MMP (Ahmad *et al.*, 2006). MT3-MMP cleaves the APP ectodomain at multiple sites that are Ala463-Met464, Asn579-Met580, His622-Ser623 and His685-Gln686, which is within the Aβ sequence (Figure 17), indicating that MT3-MMP could be an α-secretase. Since the APP fragmentation seen after cleavage by MT1-MMP, MT3-MMP and MT5-MMP was almost identical, MT1-MMP and MT5-MMP may cleave APP at the same sites as MT3-MMP (Ahmad *et al.*, 2006). As cleavage of APP by an α-secretase destroys the Aβ-sequence, shedding of APP by MT1-, MT3- and MT5-MMP might mitigate pathologic Aβ formation and accumulation.

Although familial Alzheimer's disease appears to be caused by Aβ overproduction, sporadic Alzheimer's disease (the most prevalent form) may be caused by impaired Aβ clearance. Aβ40 is a potent inducer of MMP-2, MMP-3 and MMP-9 expression *in vitro* (Deb and Gottschall, 1996). Furthermore, MMP-2 is capable of degrading Aβ40 and Aβ42 *in vitro* at the Lys16-Leu17, Leu34-Met35 and Met35-Val36 peptide bonds, although this does not lead to a complete clearing of Aβ (Roher *et al.*, 1994). Treatment of cells overexpressing APP with the metal ligand clioquinol and Cu^{2+} or Zn^{2+} resulted in an ~85 to 90% reduction of Aβ40 and Aβ42. This loss of Aβ was not caused by altered APP processing, but was mediated through upregulation of MMP-2 and MMP-3. Inhibitors of MMP-2 and MMP-3 indeed abrogated the loss of Aβ40 caused by clioquinon and Cu^{2+} (White *et al.*, 2006). However, further investigation will be necessary to determine wether MMP-2- and MMP-3-mediated cleavage is a rate-limiting step in the rapid clearance of secreted Aβ *in vitro* as well as *in vivo*. Interestingly, the human hippocampus of Alzheimer's disease patients shows increased levels of pro-MMP-9 near the amyloid plaques (Backstrom *et al.*, 1996) and the same increase was reported in amyloid-positive beagle brains (Lim *et al.*, 1997). In addition, levels of circulating MMP-9 are significantly elevated in the plasma of Alzheimer's disease patients compared

to controls (Lorenzl *et al.*, 2003). MMP-9 processes soluble Aβ40 and Aβ42 *in vitro* at multiple sites that are Lys16-Leu17; Phe20-Ala21; Asp23-Val24; Ala30-Ile31; Gly33-Leu34; Leu34-Met35 and Gly37-Gly38 (Figure 17) (Backstrom *et al.*, 1996; Yan *et al.*, 2006) However, in contrast to other proteases that also degrade sAβ such as endothelin-converting enzyme, insulin-degrading enzyme, and neprylisin, MMP-9 was the only one to degrade Aβ fibrils *in vitro*. In addition, amorphous structures suggestive of decomposed fibrils were observed after incubation with MMP-9. Fibril disruption by MMP-9 produced Aβ fragment with molecular weights corresponding to Aβ20 and Aβ30, suggesting that proteolytic cleavage at Phe20-Ala21 and Ala30-Ile31 may be important for fibril degradation. MMP-9 was also shown to degrade compact amyloid plaques in brain sections from aged APP/presenilin(PS)1 mice. Fibrillar Aβ in compact plaques is believed to be extremely resistant to degradation and clearance, but growing evidence suggests that endogenous mechanisms for plaque clearance exist, as amyloid plaque size in brains of AD patients does not invariably increase with disease duration and in aged APP/swedisch mutation(sw) mice (overpoducing APPβ), some isolated plaques even decrease in size over time. Furthermore, MMP-9 is expressed in astrocytes surrounding plaques in the brains of aged APP/PS1 mice, and its activity is specifically detected in compact plaques (Yan *et al.*, 2006). Thus, MMP-9 may contribute to clearance of plaques from amyloid-laden brains. *In vivo*, significant increases in the steady-state levels of sAβ were found in the brains of MMP-2 and MMP-9 deficient mice compared with wild-type controls. In addition, pharmacological inhibition of the MMPs with the broad-spectrum inhibitor GM6001 increased brain interstitial sAβ levels and half-life in APP/sw mice. Under these disease-free steady-state conditions, gene deletion of *mmp-2* appeared to have a greater effect on brain Aβ levels compared with *mmp-9* gene deletion (Yin *et al.*, 2006). However, it is possible that under pathological conditions, MMP-9 may play a greater role in Aβ clearance, as its expression was shown to be increased in astrocytes surrounding amyloid plaques, as mentioned above. In summary, a better understanding of the role of MMPs in the clearance of extracellular sAβ and in the degradation of Aβ fibrils in amyloid plaques, may point to alternative therapeutical approaches that reduce plaque formation and slow down the disease cascade in early stages.

4.2 FasL

The growing evidence that Aβ accumulation is a determining factor in Alzheimer's disease makes it important to elucidate the mechanism by which Aβ induces neuronal cell death. It was shown that Aβ induces neuronal cell apoptosis and that inhibition of FasL and Fas function led to a decrease in Aβ-induced neuronal apoptosis (Morishima *et al.*, 2001). As discussed before, MMP-7 releases sFasL, which is a less potent apoptosis-inducer than its membrane-bound precursor (see Section 1.2.1). Whereas treatment of neuronal cell cultures with sAβ alone increased the appearance of morphologically apoptotic cells and nuclei, addition of MMP-7 increased sFasL shedding in the culture media and compeletely protected neuron cultures from Aβ toxicity (Ethell *et al.*, 2002). Hence, FasL shedding by MMP-7 is another important MMP-mediated proteolytic process at the cell surface. Factors that affect this shedding process may play a role in the progression of Alzheimer's disease and may provide an avenue for therapeutic intervention.

5. MEMBRANE-ASSOCIATED PROTEOLYSIS IN REPRODUCTIVE ENDOCRINOLOGY

5.1 LRP

Similar to the intense tissue remodelling associated with cancer progression, the human endometrium undergoes cyclic growth and tissue remodelling throughout the reproductive life of women, with the succession of proliferative, secretory, and menstrual phases. In this rapidly changing environment, successful embryo implantation requires a tight control of the integrity of the endometrial tissue at the early and mid-secretory phase. This implies a strict control of u-PA and MMPs, which are repressed by progesterone. As discussed before (see Section 1.4.9), LRP-mediated internalization of proteases is an important regulation mechanism for proteolytic activity at the cell surface. Interestingly, the expression of LRP mRNA also varies during the menstrual cycle, with a significant increase from the proliferative to the secretory phase, when progesterone concentration is the highest, possibly promoting further repression of MMP acitivity (Emonard *et al.*, 2005). Furthermore, LRP is an important molecule during embryonic development (Herz *et al.*, 1992). LRP is highly expressed in the placenta and increased levels of soluble

LRP (sLRP) were detected in cord blood from healthy pregnancy. While sLRP release from BeWo choriocarcinoma cells is prevented by the hydroxamic acid compound, INH-38SS-PI (Quinn *et al.*, 1999), the cleavage is not mediated by MT-MMPs as it involves endoproteolysis of the membrane-spanning β-chain (LRP-85), whereas MT-MMPs cleave in the COOH-terminal part of the α-chain (LRP-515) (see Section 1.4.9 and Figure 10) (Rozanov *et al.*, 2004a). In addition, the metalloproteinase responsible for the cleavage is not induced by PMA. The BeWo cell line, which is derived from a human gestational choriocarcinoma, displays morphological and functional characteristics of both invasive cytotrophoblast and syncytiotrophoblast. The release of biologically active sLRP by trophoblast cells may have implications regarding the biology of the placenta. The pattern of LRP expression in the placenta is consistent with roles for the receptor in trophoblast invasion, a tightly regulated process that involves the coordinated activation of proteases and the transport of cholesterol. In term placenta, LRP is expressed in the syncytium, which comprises the maternal-fetal interface. Increased levels of sLRP in cord blood may reflect cellular dysfunction and increased metalloproteinase activity at this important interface (Quinn *et al.*, 1999). Hence, further investigation into the release of sLRP and its interaction with cellular LRP might be of interest to predict or monitor complications during embryonic development.

5.2 HB-EGF

Transmembrane and soluble HB-EGF have a crucial role in some events of female reproduction biology. Firstly, in blastocyst implantation, mHB-EGF, but not sHB-EGF, has been shown to promote adhesion between the blastocyst and the uterine wall, whereas sHB-EGF has been reported to induce an increase in blastocyst number and an increase in the rate of blastocyst zona pellucida hatching (Raab and Klagsbrun, 1997; Iwamoto and Mekada, 2000). Furthermore, cleavage of mHB-EGF by MMP-7 and the subsequent activation of ErbB4/HER4 by sHB-EGF appear to play a role in the regulation of postpartum uterine and lactating mammary gland involution and maintenance of lactation. CD44 plays a key role in this TMPS (by assembling MMP-7, mHB-EGF and ErbB4 in a cell surface complex (see Section 1.4.7 and Figure 9) (Yu *et al.*, 2002). Moreover, release of sHB-EGF by MMP-2 and MMP-9 is a crucial step in neuroendocrine regulation by the gonadotropin-releasing hormone (GnRH) which regulates the synthesis and secretion of the gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH) in the pituitary gland. Inhibition of MMP-2 and MMP-9 by selective inhibitors (Ro28–2653) or by specific ribozymes indeed blocks transactivation of the EGFR, induced by the binding of GnRH to its receptor (a GPCR) (Roelle *et al.*, 2003; Shah *et al.*, 2004). MT1-MMP comes out as an additional player in this TMPS through the activation of MMP-2 (Shah and Catt, 2004c). Similar to GnRH, the steroid hormone estradiol (E2) also binds GPCRs, an alternative type of estrogen receptors, and rapidly stimulates signal transduction through transactivation of the EGFR. In this TMPS sHB-EGF release is also mediated by MMP-2 and MMP-9 (Razandi *et al.*, 2003). Thus, ectodomain cleavage of HB-EGF by MMPs is a crucial event in the regulation of some neuroendocrine and reproductive functions.

5.3 Occludin

In the low-resistance human vaginal-cervical epithelia, occludin is present in two main forms: the full-length 65 kDa wild-type isoform, and a truncated 50 kDa form. A shift from 65 kDa to 50 kDa can be induced by treatment with estrogen and is associated with a reversible decrease in the resistance of the tight junctions. MMPs were already described as being responsible for

TABLE 3 **pro-TNF-α cleavage sites**

Protease	Cleavage site(s)	Test system	References
TACE	Ala76-Val77	*In vitro* and in vivo	3
MMP-1	Ala74-Gln75 and Ala76-Val77	*In vitro*	1,2,3
MMP-2	Not defined	*In vitro*	1,2
MMP-3	Not defined	*In vitro*	1,2,3
MMP-7	Ala76-Val77	*In vitro* and ex vivo	1,2,3,6
MMP-9	Ala74-Gln75	*In vitro*	1,2,3
MMP-12	Ala74-Gln75 and Ala76-Val77	*In vitro* and in vivo	7,8
MT1-MMP	Ala76-Val77 and before Leu113	*In vitro*	4
MT2-MMP	Ala76-Val77 and before Leu113	*In vitro*	4
MT4-MMP	Ala74-Gln75	*In vitro*	5

1, (Gearing *et al.*, 1994); 2, (Gearing *et al.*, 1995); 3, (Mohan *et al.*, 2002); 4, (d'Ortho *et al.*, 1997);
5, (English *et al.*, 2000); 6, (Haro *et al.*, 2000b); 7, (Chandler *et al.*, 1996); 8, (Churg *et al.*, 2003).

TABLE 4 **Potential membrane-bound MMP and MP substrates**

Substrate[1]	CD	Inhibitor	Specificity[2]	Reference
ACE	CD143	EDTA, O-phen	MP	(Parvathy *et al.*, 1997)
		Batimastat (BB-94)	Broad spectrum MMP and TACE	
		TAPI-2	Zn^{2+}-dependent MP	
		TAPI(-1)	Collagenase, gelatinase, ADAM-10, TACE	(Schwager *et al.*, 1998; Schwager *et al.*, 1999)
ANF-R	/	EDTA	MP	(Abe and Misono, 1992)
AR	/	Batimastat (BB-94)	Broad spectrum MMP and TACE	(Vecchi *et al.*, 1998; Brown *et al.*, 1998)
C1qRp	CD93	O-phen	MP	(Bohlson *et al.*, 2005)
		Not inhibited by: TAPI-1, TAPI-2 and the broad spectrum MMP inhibitors Ro-31–9790, Ro-32–7315		
CD27	CD27	GI5402	MMP-1,-3,-9,-13 and TACE	(Dekkers *et al.*, 2000)
CD30	CD30	EDTA, O-phen	MP	(Parvathy *et al.*, 1997) (Hansen *et al.*, 1995; Hooper *et al.*, 1997)
		BB2116	MMP-3, -7, -2, -9	
CD40L	CD154	EDTA	MP	(Furman *et al.*, 2004; Otterdal *et al.*, 2004)
		Ilomastat (GM6001)	Broad spectrum MMP and TACE	
		KB8301	Zn^{2+}-dependent MP	(Kato *et al.*, 1999; Jin *et al.*, 2001)
		Batimastat (BB-94)	Broad spectrum and TACE	
c-Met	/	TIMP-3	Broad spectrum MMP ADAM-10, -12, -17, -19 ADAMTS-4 and -5	(Nath *et al.*, 2001)
		Batimastat (BB-94)	Broad spectrum MMP and TACE	
		Not inhibited by TIMP-1 and TIMP-2		
CXCL16	/	Ilomastat (GM6001)	Broad spectrum MMP and TACE	(Hara *et al.*, 2006)
		MMP inhibitor III*	Broad spectrum MMP	
Desmocollin-3	/	FN-439 (MMP inhibitor I*)	MMP-1, MMP-8 > MMP-9 > MMP-3	(Weiske *et al.*, 2001)
		Not inhibited by TAPI(-1)		
Desmoglein-1	/	TAPI-0	Collagenase, gelatinase, TACE	(Dusek *et al.*, 2006)
		FN-439 (MMP inhibitor I*)	MMP-1, MMP-8 > MMP-9 > MMP-3	
		Ilomastat (GM6001)	Broad spectrum MMP and TACE	
Desmoglein-3	/	FN-439 (MMP inhibitor I*)	MMP-1, MMP-8 > MMP-9 > MMP-3	(Weiske *et al.*, 2001)
		Not inhibited by TAPI(-1)		
EGF	/	EDTA, EGTA	MP	(Dempsey *et al.*, 1997)
		Batimastat (BB-94)	Broad spectrum MMP and TACE	
FcγRIII	CD16	GI5402	MMP-1,-3,-9,-13 and TACE	(Dekkers *et al.*, 2000)
		O-phen	MP	(Bazil and Strominger, 1994) (Galon *et al.*, 1998)
		RU36156	MMP-8, MMP-9 and TACE	(Galon *et al.*, 1998; Mota *et al.*, 2004)

TABLE 4 **Potential membrane-bound MMP and MP substrates *(Continued)***

Substrate[1]	CD	Inhibitor	Specificity[2]	Reference
FcεRII	CD23	O-phen	MP	(Bailey *et al.*, 1998)
		Batimastat (BB-94)	Broad spectrum MMP and TACE	
Folate-R	/	EDTA, O-phen	MP	(Elwood *et al.*, 1991)
GHR	/	BB-3103	Broad spectrum MMP and TACE	(Amit *et al.*, 2001)
		Ro31-9790	Broad spectrum MMP and TACE	
GM-CSF-Rα	CD116	Batimastat (BB-94)	Broad spectrum MMP and TACE	(Prevost *et al.*, 2002)
GP VI	/	TAPI(-1)	Collagenase, gelatinase, ADAM-10, TACE	(Stephens *et al.*, 2005)
		Ilomastat (GM6001)	Broad spectrum MMP and TACE	
HER2	CD340	TIMP-1	Soluble MMPs	(Codony-Servat *et al.*, 1999; Molina *et al.*, 2001)
		TAPI(-1)	Collagenase, gelatinase, ADAM-10, TACE	
		Batimastat (BB-94)	Broad spectrum MMP and TACE	
HER4	/	Batimastat (BB-94)	Broad spectrum MMP and TACE	(Vecchi *et al.*, 1998)
IL-1RII	CD121b	Batimastat (BB-94)	Broad spectrum MMP and TACE	(Penton-Rol *et al.*, 1999)
IL-4R	CD124	O-phen	MP	(Jung *et al.*, 1999)
IL-6Rα	CD126	TAPI(-1)	Collagenase, gelatinase, ADAM-10, TACE	(Mullberg *et al.*, 1995)
		RU36156	MMP-8, MMP-9 and TACE	(Gallea-Robache *et al.*, 1997)
		TAPI-2	Zn^{2+}-dependent MP	(Arribas *et al.*, 1996)
		O-phen	MP	
LDL-R	/	EDTA, EGTA	MP	(Begg *et al.*, 2004)
		TAPI(-1)	Collagenase, gelatinase, ADAM-10, TACE	
Leukosialin	CD43	O-phen	MP	(Bazil and Strominger, 1994)
LIGHT	CD258	EDTA	MP	(Otterdal *et al.*, 2006)
		Ilomastat (GM6001)	Broad spectrum MMP and TACE	
M-CSF	/	RU36156	MMP-8, MMP-9 and TACE	(Gallea-Robache *et al.*, 1997)
Megalin	/	MMP inhibitor III*	Broad spectrum MMP	(Zou *et al.*, 2004)
		TAPI(-1)	Collagenase, gelatinase, ADAM-10, TACE	
		TAPI-2	Zn^{2+}-dependent MP	
MICA	/	Batimastat-derivative	Zn^{2+}-dependent MP	(Salih *et al.*, 2002)
MICB	/	Batimastat-derivative	Zn^{2+}-dependent MP	(Salih *et al.*, 2006)
MMR-1	CD206	BB2116	MMP-3, -7, -2, -9	(Martinez-Pomares *et al.*, 1998)
N-CAM L1	CD171	BB-3103	Broad spectrum MMP and TACE	(Mechtersheimer *et al.*, 2001)
		Ro-31-9790	Broad spectrum MMP and TACE	
		TAPI(-1)	Collagenase, gelatinase, ADAM-10, TACE	(Beer *et al.*, 1999; Gutwein *et al.*, 2005)
Nectin-1α	CD111	O-phen	MP	(Tanaka *et al.*, 2002)
		Batimastat (BB-94)	Broad spectrum MMP and TACE	
		KB-R7785	MMP-1,-2,-3,-9,-14 TACE, ADAM12	
Netrin-1	/	Ilomastat (GM6001)	Broad spectrum MMP and TACE	(Galko and Tessier-Lavigne, 2000)
		TAPI-2	Zn^{2+}-dependent MP	

(Continued on next page)

TABLE 4 **Potential membrane-bound MMP and MP substrates *(Continued)***

Substrate[1]	CD	Inhibitor	Specificity[2]	Reference
NGF-R	/	EDTA, O-phen	MP	(Diaz-Rodriguez *et al.*, 1999)
		TAPI-2	Zn^{2+}-dependent MP	
NKR BY55	CD160	O-phen	MP	(Giustiniani *et al.*, 2007)
PECAM-1	CD31	Ilomastat (GM6001)	Broad spectrum MMP and TACE	(Ilan *et al.*, 2001)
		MMP-2/MMP-9 Inhibitor I*	MMP-2, MMP-9	
		NNGH (MMP-3 Inhibitor II*)	MMP-3	
		MMP-8 Inhibitor*	MMP-8	
PSGL-1	CD162	EDTA Not inhibited by: O-phen, Batimastat and Marimastat	MP	(Davenpeck *et al.*, 2000)
Sialophorin	CD43	O-phen	MP	(Bazil and Strominger, 1994)
SorLA	/	BB-3103	Broad spectrum MMP and TACE	(Hampe *et al.*, 2000)
SRCR M130	CD163	TAPI-0	MMP-1, MMP-9, TACE	(Hintz *et al.*, 2002)
Syndecan-3	/	BB-3103	Broad spectrum MMP and TACE	(Asundi *et al.*, 2003)
		Batimastat (BB-94)	Broad spectrum MMP and TACE	
pro-TGF-α	/	RU36156	MMP-8, MMP-9 and TACE	(Gallea-Robache *et al.*, 1997)
		TAPI-2	Zn^{2+}-dependent MP	(Arribas *et al.*, 1996)
		TAPI(-1)	Collagenase, gelatinase, ADAM-10, TACE	(Shao *et al.*, 2004)
		O-phen	MP	(Arribas *et al.*, 1996)
		Batimastat (BB-94)	Broad spectrum MMP and TACE	(Merlos-Suarez *et al.*, 2001)
		Ilomastat (GM6001)	Broad spectrum MMP and TACE	(Yoshisue and Hasegawa, 2004; Shao *et al.*, 2004)
		Marimastat (BB-2516)	Broad spectrum MMP and TACE	
Tie-1	/	EGTA	Ca^{2+}-dependent MP	(Yabkowitz *et al.*, 1999)
		BB-24 Not inhibited by: TIMP-2	Zn^{2+}-dependent MP	
TNF-R55/60	CD120a	TAPI(-1)	Collagenase, gelatinase, ADAM-10, TACE	(Mullberg *et al.*, 1995)
		RU36156	MMP-8, MMP-9 and TACE	(Gallea-Robache *et al.*, 1997)
		BB-2275	Zn^{2+}-dependent MP	(Williams *et al.*, 1996)
TNF-R75/80	CD120b	TAPI(-1)	Collagenase, gelatinase, ADAM-10, TACE	(Crowe *et al.*, 1995)

TABLE 4 **Potential membrane-bound MMP and MP substrates *(Continued)***

Substrate[1]	CD	Inhibitor	Specificity[2]	Reference
		RU36156	MMP-8, MMP-9 and TACE	(Gallea-Robache *et al.*, 1997)
TSHR	/	BB2116	MMP-3, -7, -2, -9	(Couet *et al.*, 1996; Misrahi and Milgrom, 1997; de Bernard *et al.*, 1999)
		Not inhibited by TIMP-1, TIMP-2		(de Bernard *et al.*, 1999)
ULBP2	/	Batimastat (BB-94)	Broad spectrum MMP and TACE	(Waldhauer and Steinle, 2006)
		MMP inhibitor III*	Broad spectrum	(Waldhauer and Steinle, 2006)
VCAM-1	CD106	Marimastat (BB-2516)	Broad spectrum MMP and TACE	(Hummel *et al.*, 2001)
VPR V2	/	O-phen	MP	(Kojro and Fahrenholz, 1995)

[1]Substrate acronyms: ACE, angiotensin-converting enzyme; ANF-R, atrial natriuretic factor receptor; C1qRp, complement component 1 q subcomponent receptor 1; AR, amphiregulin; c-Met, met proto-oncogene TKR; CXCL16, transmembrane chemokine CXCL16; FcγRIII, low affinity Ig γ Fc receptor III; FcεRII, low affinity Ig ε Fc receptor II; GHR, growth hormone receptor; GM-CSF-Rα, granulocyte-macrophage colony-stimulating factor receptor α chain; GP VI, glycoprotein VI; HER4, Tyrosine kinase-type cell surface receptor HER4; IL -R, interleukin receptor; LDL-R, low-density lipoprotein receptor; LIGHT, homologous to lymphotoxins, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for herpes virus entry mediator, a receptor expressed by T lymphocytes; M-CSF, macrophage-colony stimulating factor; MICA/B, MHC class I chain-related gene A/B; MMR-1, Macrophage mannose receptor-1; N-CAM L1, neural cell adhesion molecule L1; NGF-R, nerve growth factor receptor; NKR BY55, NK cell receptor BY55; NNGH, N-Isobutyl-N-(4-methoxyphenylsulfonyl)-glycylhydroxamic acid; O-phen, 1,10-phenantroline; PECAM-1, platelet endothelial cell adhesion molecule-1; PSGL-1, P-selectin glycoprotein ligand-1; -R, receptor; SorLA, sorting protein-related receptor containing LDL-R class A repeats; SRCR M130, scavenger receptor cysteine-rich type 1 protein M130; TAPI, TNF-α Protease Inhibitor; Tie-1, tyrosine kinase receptor tie-1; TSHR, thyrotropin receptor; ULBP2, UL16-binding protein 2; VCAM-1, Vascular cell adhesion molecule-1; VPR V2, V2 vasopressin receptor. Additional acronyms can be found in the list of abbreviations.

[2]Inhibitor specificities: for detailed information, the reader is referred to the reviews (Kontogiorgis *et al.*, 2005), (Sang *et al.*, 2006) and (Whittaker *et al.*, 1999).

*Additional information on the inhibitor structure and characteristics can be found at http://www.merckbiosciences.co.uk/home.asp.

occludin degradation and increased permeabilization of endothelial cell and epithelial sheets (see Section 2.2 and Figures 14 to 16). Treatment of human normal vaginal-cervical cells with natural doses of 17β-estradiol upregulated activation of MMP-7 intracellularly, in the Golgi network, and augmented secretion of activated MMP-7. This MMP-7 was shown to be necessary and sufficient to produce estrogen-mediated decrease of tight junctional resistance and extracellular modulation of occludin. Hence, MMP-7-mediated proteolysis of occludin might be an essential step in the estrogen modulation of paracellular permeability *in vivo* (Gorodeski, 2007).

6. POTENTIAL CELL SURFACE-ASSOCIATED MMP SUBSTRATES

Processing of various membrane-bound molecules is inhibited by metalloproteinase-specific inhibitors, although the involved metalloproteinase has not been identified yet. These substrates have been grouped in Table 4 with the protease inhibitor profiles and the inhibitor specificities. As discussed before, the inhibition pattern of a proteolytic process yields information on the involved enzyme(s) or protease class(es). Ectodomain shedding of a whole array of membrane-bound molecules is inhibited by the non-specific cation chelators EDTA, EGTA, and 1,10-phenantroline (*e.g.*, ANF-R, IL-4R, leukosialin). It is relevant to notice that the affinity of EDTA is higher for light metal cations, whereas 1,10-phentroline favours the binding of heavy metal cations. In addition, EGTA preferentially chelates Ca^{2+} ions, thus inhibiting Ca^{2+}-dependent proteases. Broad-spectrum MMP inhibitors, such as most hydroxamates, also inhibit TACE and other ADAMs. Hence, a shedding process inhibited by batimastat, marimastat, GM6001, *etc.* is not necessarily mediated by an MMP and requires further investigation of the protease(s) in charge (*e.g.*,desmoglein-1, HER4, megalin, VCAM-1). Along this line, inhibition by TIMPs does not guarantee that the cleaved molecule is an MMP substrate, as

TABLE 5 **Cell surface MMP substrates without transmembrane domain**

Substrate	Type of cell membrane association	Cleaving MMP	Reference
ADAMTS-4	Association with chondroitin and heparan sulfate chains on syndecan-1	MMP-9, MMP-13	(Tortorella *et al.*, 2005)
		MT4-MMP	(Gao *et al.*, 2002; Gao *et al.*, 2004)
C3b	Amide and ester bonds with cell surface molecules	MT1-MMP	(Rozanov *et al.*, 2004b)
C4b	Amide and ester bonds with cell surface molecules	MT1-MMP	(Rozanov *et al.*, 2004b)
Galectin-3	Binding to IgE and multiple cell surface molecules	MMP-2, MMP-9	(Ochieng *et al.*, 1994; Ochieng *et al.*, 1998)
Galectin-9	Binding to cell surface molecules	ND MMP	(Chabot *et al.*, 2002)
KiSS protein	Complex with pro-MMP-2,-9	MMP-2,-9	
		MT1-, MT3-, MT5-MMP	(Takino *et al.*, 2003)
pro-TGF-β	ECM association	MMP-2,-3,-9	
		MT1-MMP	(Yu and Stamenkovic, 2000)
tTG	Binding to integrins	MT1-MMP, MMP-2	
		MT2-, MT3-MMP	(Belkin *et al.*, 2001; Belkin *et al.*, 2004)
ApoE	Complex with pro-MMP-2	MT1-MMP, MMP-7	(Aoki *et al.*, 2005)
gC1qR	Binding to the hemopexin domain of MT1-MMP	MT1-MMP	(Rozanov *et al.*, 2002)

apoE, apolipoprotein E; **C3b**, complement component 3b; **C4b**, complement component 4b; **gC1qR**, receptor of complement component 1q.

TIMP-3 inhibits various ADAMs and some proteases of the ADAMTS family (see Table 1). However, some conclusions can be drawn from a TIMP inhibition pattern. As TIMP-2 and TIMP-4 only inhibit MMPs, inhibition or absence of inhibition by these TIMPs, respectively, identifies or rejects MMPs as the operating sheddases (*e.g.*,c-Met, tie-1, TSHR). A metalloprotease inhibited by TIMP-1 is likely to be an (MT-)MMP, as ADAM-10 is the only non-MMP to be inhibited (*e.g.*,HER2). Thus, as long as few specific MMP inhibitors exist, particular care must be taken when drawing conclusions about the identity of the sheddase. Additional cell surface molecules that are modulated by ectodomain shedding have been described (Hooper *et al.*, 1997; Blobel, 2000; Dello and Rovida, 2002; Arribas and Borroto, 2002; Garton *et al.*, 2006).

Finally, some MMP substrates without transmembrane domain are located at the cell surface by receptor binding, by a membrane vesicle, a membrane-bound proteoglycan or another transmembrane protease. For that reason, these have not all been discussed in detail in the above survey. Some examples of such cell surface-associated proteins are galectin-3 and -9 (Ochieng *et al.*, 1994; Ochieng *et al.*, 1998; Chabot *et al.*, 2002); tTG (Belkin *et al.*, 2001; Belkin *et al.*, 2004); the KiSS protein/metastin (Takino *et al.*, 2003); pro-TGF-β (Yu and Stamenkovic, 2000); ADAMTS-4 (Gao *et al.*, 2004); the complement components C3b and C4b (Rozanov *et al.*, 2004b); apolipoprotein E (Aoki *et al.*, 2005) and gC1qR, the receptor of complement component 1q (Rozanov *et al.*, 2002) (Table 5).

CONCLUSION

Due to the rapid development of innovative biochemical techniques and the expanding use of transgenic and knockout animals, it became obvious that the action radius of MMPs is not restricted to massive ECM destruction in physiological tissue remodelling and pathological tissue degradation. Identification of specific matrix– as well as non-matrix–components as MMP substrates showed that MMPs also play significant roles in highly complex processes such as the regulation of cellular behavior, cell-cell communication and tumor progression (McCawley and Matrisian, 2001). An extended variety of bioactive molecules is modified by MMPs in particular physiopathological processes (Sternlicht and Werb, 2001).

The above survey points out that MMPs do not only cleave multiple soluble substrates, but also process a

whole array of membrane-bound proteins. These proteolytic events on the cell surface may have extremely diverse biological implications (see Table 2), ranging from maturation (*e.g.*, pro-α integrin subunits), activation (*e.g.*, PAR1) and potentiation (HER2) of a cell surface component, to its inactivation (*e.g.*, syndecan-1) or even its degradation (*e.g.*, NG2 proteoglycan and β-dystroglycan). Besides the regulation of cell-bound activity, ectodomain proteolysis of substrates may also be required for the diffusion of the reaction product(s) into the extracellular environment to facilitate receptor activation on adjacent or even more distal cells (*e.g.*, pro-TNF-α and RANKL). In addition, a liberated receptor fragment might as well possess inhibitory power by acting as a soluble decoy receptor that binds soluble ligands, preventing their interaction with the cell-bound, signal-transducing receptor (*e.g.*, FGFR-1).

Through these distinct effects on the biological activity of the substrates, proteolysis of cell surface proteins by MMPs has a major impact on a multitude of physiological functions, as well as on onset and evolution of many diseases. In addition, MMP activity itself can be directly regulated by proteolysis of cell surface proteins. For instance, MMPs cleave their membrane-bound inducer, EMMPRIN, allowing the active fragment to induce MMP activity in adjacent cells or in more distal tissues. Conversely, they also modulate their own internalization and degradation by degrading their scavenger receptor, LRP, assuring sustained MMP activity. Finally, MMPs can terminate their own activity by autocatalytic proteolysis.

Inhibition of MMPs seems to be the ideal solution in many pathologies. However, the enthusiasm generated by a large number of *in vitro* and *in vivo* studies has been dramatically mitigated in recent years by the failure of MMP inhibitors to block tumor progression in clinical trials (Coussens *et al.*, 2002). This fiasco could be explained partly by the fact that inhibitors were administered only to late-stage cancer patients, whereas proteases are often involved in early stages of tumor progression. In addition, the lack of selectivity and specificity of inhibitors is a problem, as tumor invasion and metastasis require the concerted action of particular MMPs. However, as stated by Del Rosso and coworkers (2005), our understanding of protease-environment interactions is far from exhaustive. The initial, naïve view of proteases acting in the soluble phase has evolved into a kaleidoscope of images in which proteolytic reactions in tissue remodelling mostly occur at, and are modulated by, the cell surface. As a consequence, insight in the modification of cell surface proteins by MMPs and the associated implications, is essential to elucidate the cross-talk between proteases at the cell surface and with the extracellular environment. In conclusion, starting with the exploration of the cell surface could become a prerequisite in the succesful development of new MMP inhibitors and innovative therapeutic approaches for cancer and inflammatory diseases.

ABBREVIATIONS

Aβ, β-amyloid protein; **ACE,** angiotensin-converting enzyme; **ADAM,** a disentegrin and metalloproteinase; **ADAMTS,** a disentegrin and metalloproteinase with thrombospondin-like motif; **ANF-R,** atrial natriuretic factor receptor; **AP,** alkaline phosphatise; **ApoE,** apolipoprotein E; **APP,** amyloid precursor protein; **AR,** amphiregulin; **ARF,** acute renal failure; **BBB,** blood-brain barrier; **BP,** bullous pemphigoid; **BP-180,** BP antigen-2; **BRB,** blood-retinal barrier; **C1qRp,** complement component 1 q subcomponent receptor 1; **C3b/4b,** complement component 3b/4b; **CD,** cluster of differentiation of human (glyco)proteins; **CD44ICD,** intracellular domain of CD44; **c-Met**, met proto-oncogene TKR; **CNS,** central nervous system; **CTL,** cytotoxic T cell; **EAE,** experimental autoimmune encephalomyelitis; **E-cadherin,** epithelial cadherin; **ECM,** extracellular matrix; **EDTA,** ethylenediaminetetraacetic acid; **EGF(R),** epidermal growth factor (receptor); **EGTA,** ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid; **EMMPRIN,** extracellular matrix metalloproteinase inducer; **FasL,** Fas ligand; **FcyRIII,** low affinity Ig γ Fc receptor III; **FcεRII,** low affinity Ig ε Fc receptor II; **FGF,** fibroblast growth factor; **FGFR-1,** FGF receptor-1; **gC1qR,** receptor of complement component 1q; **GHR,** growth hormone receptor; **GM-CSF-Rα,** granulocyte-macrophage colony-stimulating factor receptor α chain; **GnRH,** gonadotropin-releasing hormone; **GP VI,** glycoprotein VI; **GPCR,** G protein-coupled receptor; **GPI,** glycosyl phosphatidylinositol; **HB-EGF,** heparin-binding epidermal growth factor-like growth factor; **HER2/4,** tyrosine kinase-type cell surface receptor HER2/4; **ICAM-1,** intercellular adhesion molecule-1; **ICE,** IL-1β-converting enzyme; **Ig,** immunoglobulin; **IL- (R),** interleukin- (receptor); **IL-1β,** interleukin-1β; **KitL,** Kit ligand; **KS,** keratoconjunctivitis sicca; **LDL(-R),** low density lipoprotein (receptor); **LIGHT,** homologous to

lymphotoxins, exhibits **i**nducible expression, and competes with herpes simplex virus **g**lycoprotein D for **h**erpes virus entry mediator, a receptor expressed by **T** lymphocytes; **LPS,** lipopolysaccharide; **LR,** 34/67 kDa laminin receptor; **LRP,** low-density lipoprotein receptor-related protein; **L-selectin,** leukocyte-selectin; **m-,** membrane-bound; **MBP,** myelin basic protein; **M-CSF,** macrophage-colony stimulating factor; **MEVC,** microvascular endothelial cell; **MICA/B,** MHC class I chain-related gene A/B; **MMP,** matrix metalloproteinase; **MMR-1,** macrophage mannose receptor-1; **MP,** metalloproteinase; **MT-MMP,** membrane-type MMP; **MUC1,** mucin-1; **N-cadherin,** neuronal cadherin; **N-CAM L1,** neural cell adhesion molecule L1; **NE,** neutrophil elastase; **NGF-R,** nerve growth factor receptor; **NK cell,** natural killer cell; **NKR BY55,** NK cell receptor BY55; **PAR1,** protease-activated receptor-1; **PC,** proprotein convertase; **PDGF,** platelet-derived growth factor; **PDR,** proliferative diabetic retinopathy; **PECAM-1,** platelet endothelial cell adhesion molecule-1; **PLAD,** preligand assembly domain; **PMA,** phorbol 12-myristate 13-acetate; **PSGL-1,** P-selectin glycoprotein ligand-1; **PVR,** proliferative vitreoretinopathy; **-R,** receptor; **RANK(L),** receptor activator of nuclear factor κB (ligand); **s-,** soluble; **SDF-1,** stromal cell-derived factor-1; **SorLA,** sorting protein-related receptor containing LDL-R class A repeats; **SP-D,** surfactant protein-D; **SRCR M130,** scavenger receptor cysteine-rich type 1 protein M130; **Ssc,** systemic sclerosis; **TACE,** TNF-α-converting enzyme; **TAPI,** TNF-α protease inhibitor; **TGF-α/β,** transforming growth factor-α/β; **Tie-1 ,** tyrosine kinase receptor tie-1; **TIL,** tumor infiltrating lymphocyte; **TIMP,** tissue inhibitor of metalloproteinases; **TKR,** tyrosine kinase receptor; **TMD,** transmembrane domain; **TMPS,** triple membrane-passing signal mechanism; **TNF-α,** tumor necrosis factor-α; **t-PA,** tissue-type plasminogen activator; **TSHR,** thyrotropin receptor; **tTG,** tissue transglutaminase; **ULBP2,** UL16-binding protein 2; **u-PA,** urokinase-type plasminogen activator; **uPAR,** urokinase-type plasminogen activator receptor; **VCAM-1,** vascular cell adhesion molecule-1; **VE-cadherin,** vascular endothelial-cadherin; **VEGF,** vascular endothelial growth factor; **VPR V2,** V2 vasopressin receptor; ***X,*** *Xenopus*.

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CHAPTER 2. ADENYLYL CYCLASE-ASSOCIATED PROTEIN-1/CAP1 AS A BIOLOGICAL TARGET SUBSTRATE OF GELATINASE B/MMP-9

Cauwe B, Martens E, Van den Steen PE, Proost P, Van Aelst I, Blockmans D and Opdenakker G.

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HIGHLIGHTS

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Adenylyl cyclase-associated protein-1/CAP1 as a biological target substrate of gelatinase B/MMP-9. By Bénédicte Cauwe, Erik Martens, Philippe E. Van den Steen, Paul Proost, Ilse Van Aelst, Daniel Blockmans, and Ghislain Opdenakker. . . . . .

Matrix metalloproteinases (MMPs) are notoriously known for their tissue remodeling functions as well as for their regulatory "processing" of cytokines, protein hormones, chemokines and numerous cell membrane-bound molecules. Above and beyond these extracellular substrates, the article by Cauwe *et al.* now shows that gelatinase B/MMP-9 exerts a novel role of MMPs by the degradation of intracellular proteins during cytolysis. This was illustrated by the identification of the cytoskeletal protein "adenylyl cyclase-associated protein-1" as a novel MMP-9 substrate *in vitro*, and *in vivo* in patients with systemic autoimmune diseases. These findings suggest that intracellular substrates may be proteolyzed into "remnant neo-epitopes," in this way initiating or exacerbating systemic autoimmunity. Alternatively, degradation of abundant cytoskeletal proteins may be critical to avoid (actin) toxicity after extensive cell death.

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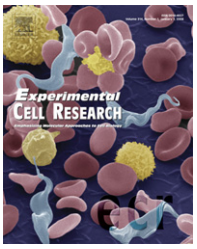

Research Article

Adenylyl cyclase-associated protein-1/CAP1 as a biological target substrate of gelatinase B/MMP-9

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ABSTRACT

Matrix metalloproteinases (MMPs) are classically associated with the turnover of secreted structural and functional proteins. Although MMPs have been shown to process also a kaleidoscope of membrane-associated substrates, little is known about the processing of intracellular proteins by MMPs. Physiological and pathological cell apoptosis, necrosis and tumor lysis by chemotherapy, radiotherapy or immunological cytotoxicity, are examples of conditions in which an overload of intracellular proteins becomes accessible to the action of MMPs. We used a model system of dying human myelomonocytic cells to study the processing of intracellular protein substrates by gelatinase B/MMP-9 *in vitro*. Adenylyl cyclase-associated protein-1 or CAP1 was identified as a novel and most efficient substrate of gelatinase B/MMP-9. The presence of CAP1 in the extracellular milieu *in vivo* was documented by analysis of urine of patients with systemic autoimmune diseases. Whereas no active MMP-9 could be detected in urines of healthy controls, all urine samples of patients with clinical parameters of renal failure contained activated MMP-9 and/or MMP-2. In addition, in some of these patients indications of CAP1 cleavage are observed, implying CAP1 degradation *in vivo*. The high turnover rate of CAP1 by MMP-9, comparable to that of gelatin as the natural extracellular substrate of this enzyme, may be critical to prevent pathological conditions associated with considerable cytolysis.

Introduction

Matrix metalloproteinases or MMPs are notably known for their cleavage of extracellular matrix components. In addition, they play critical regulatory functions by the proteolytic release of signaling molecules and by the modification of the biological functions of e.g. hormones, cytokines and chemokines [1]. With the development of knock-out and knock-in mice, recombinant MMPs, and selective inhibitors, it becomes possible to determine biologically relevant substrates of particular MMPs [2]. In this way, a whole range of membrane-associated substrates, including adhesion molecules, mediators of apoptosis, receptors, chemokines, cytokines, growth factors, proteases, intercellular junction proteins, and structural molecules has been identified [3]. An unanswered question is whether intracellular proteins are biological MMP substrates, i.e. whether conditions exist in which such proteolysis

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is useful or necessary to maintain the integrity of the host. When tissues or cells are damaged by trauma or other stress factors, such as mechanical (crush lesions), thermal (burns and frost), infections (viruses), inflammatory (reactive oxygen intermediates), (bio-) chemical (toxins), oxygen or nutrient deprivation, or even genetic factors (e.g. cytotoxicity in storage diseases), necrosis or apoptosis mechanisms initiate a cascade of events leading to degradation of cellular macromolecules and macromolecular complexes, and to recycling mechanisms. In all these conditions extracellular proteases in the stroma or other body compartments may access intracellular substrates. Similarly, during normal physiological processes such as development, thymic selection and when endogenous cancer cells are eliminated by cytotoxic natural killer cells or T lymphocytes, the complete set of (glyco)proteins, including intracellular ones, must be degraded. Intracellular MMP substrates have been found in some studies, but the physiological or pathological roles of these cleavages have not always been clarified. Examples are the proteolysis of the intraneuronal protein SNAP-25 by MMP-7 [4] and the cleavage of poly-(ADP-ribose)-polymerase (PARP) by MMP-2 [5]. An interesting group of intracellular MMP substrates are the crystallins, some of which are associated with multiple sclerosis [6] or with lens cataract development [7]. In the example of αB-crystallin in association with multiple sclerosis, the concept that this molecule acts as a functional protective factor has recently been reinforced by genetic knock-out studies in mice [8]. The example of lens crystallins illustrates that intracellular structural proteins may be MMP substrates. For these reasons, the further search for intracellular substrates (structural and functional ones) is amongst the next challenges for MMP research. Here, we provide cell biological, biochemical and *in vivo* evidences for this concept and identify a cytoskeletal protein as one of the most efficient MMP-9 substrates.

Materials and methods

Cell culture

The human myelomonocytic cell line THP-1 was cultured in RPMI 1640 medium (Bio Whittaker Europe, Verviers, Belgium), supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, California, USA) and 1.5% sodium bicarbonate. Prior to the experiments, cells were washed three times and resuspended in serum-free RPMI 1640 with 1.5% sodium bicarbonate at a concentration of 1×10^6 cells/ml or 10×10^6 cells/ml in multi-well dishes (Nunc, Roskilde, Denmark).

Induction of cytolysis in THP-1 cells and detection by flow cytometry

THP-1 cells were resuspended at a concentration of 1×10^6 cells/ml in serum-free medium and incubated for 40 h at 37 °C in the absence or presence of a general proteinase inhibitor cocktail (PIn, Complete Mini EDTA-free, Roche, Basel, Switzerland) (1 tablet/10 ml), 10 mM 1,10-*o*-phenanthroline (O-Phen, Sigma, St. Louis, MO, USA) and 17 μM Z-Val-Ala-Asp.fluoromethylketone (Z-VAD.fmk, Sigma). Alternatively, cells were resuspended at 10×10^6 cells/ml and incubated in serum-free conditions for 4.5 h at 37 °C. After incubation, cells and culture media were separated by centrifugation and culture media were stored at −20 °C. 10^6 cells per condition were washed with phosphate buffered saline (PBS) and stained with 100 μl of Annexin-V-fluorescein (FITC) and Propidium iodide labeling solution (Annexin-V-FLUOS Staining kit, Roche) according to the manufacturer's recommendations. Flow-cytometric analysis was performed on a FACSCalibur™ flow cytometer and data were processed with the Cell Quest® software (Becton Dickinson, San Jose, CA, USA).

Preparation of THP-1 cell extracts and cytosolic fractions

Cell pellets obtained after 4.5 h incubation in serum-free conditions were washed twice and resuspended at the original concentration (10×10^6 cells/ml) in reducing SDS-PAGE loading buffer (0.125 M Tris/HCl, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.05% bromophenol blue). After sonication and centrifugation of the cell extracts, the resulting supernatant was analyzed by SDS-PAGE. For the cytosol extraction, 600×10^6 cells were washed three times with assay buffer and resuspended in 6 ml assay buffer (100 mM Tris/HCl, pH 7.4, 100 mM NaCl, 10 mM $CaCl_2$) supplemented with 0.01% Tween-20 and a general proteinase inhibitor cocktail (PIn, Complete Mini EDTA-free, Roche) (1 tablet/10 ml). The cells were lysed by 4 freeze-thaw cycles and DNA was sheared by sonication. Cytosol and membrane fractions were separated by ultracentrifugation at 110,000 g with a swing-out rotor SW50.1 in a Beckman L7-55 Ultracentrifuge (Beckman Coulter, Fullerton, CA, USA) during 75 min at 4 °C. The resulting supernatant or cytosolic fraction was kept at −20 °C for further analysis.

Purification and activation of recombinant human MMPs

Recombinant human MMP-9 and MMP-9 domain deletion mutants were expressed in Sf9 insect cells and purified by gelatin-Sepharose affinity chromatography as described previously [9,10]. The purified MMP-9 variants (10 μM) were activated with 0.1 μM of the catalytic domain of human MMP-3 (cd-MMP-3, Calbiochem, Darmstadt, Germany) for 1.5 h at 37 °C in assay buffer supplemented with 0.001% Tween-20 (Sigma). MMP-1, MMP-8 and MMP-13 were activated with APMA (*p*-aminophenylmercuric acetate) according to the instructions of the manufacturer (R&D Systems, Abingdom, UK). The commercial preparation of MMP-2 (R&D systems) was found to be active without activation and therefore used as such.

Incubation of THP-1 cells or cytosol with activated MMP-9

THP-1 cytolysis was induced as described above. Before the start of induction, 1 μM activated MMP-9 was added to the cells (10×10^6 cells/ml). As a control, cells were incubated without MMP-9 or in the presence of equivalent amounts of the activator, cd-MMP-3 (10 nM). An additional control consisted of MMP-9 incubated at equivalent concentration (1 μM) in serum-free medium. After 4.5 h of incubation, culture media were harvested by centrifugation and stored at −20 °C. To assess cleavage efficiency and inhibition profiles of the major substrate(s), culture medium of THP-1 cells, harvested after 4.5 h of cytolysis induction was used as source of substrate(s). This culture medium was incubated for 4.5 h in the presence of the indicated concentrations of MMP-9 and cd-MMP-3, as well as with addition of 20 mM EDTA, 33 μg/ml TIMP-1 (R&D

Systems), 10 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (Pefabloc®, Roche), 50 mM benzamidine (Sigma) and 2 μg/ml E64 (Sigma). THP-1 cytosol was incubated for the indicated time intervals in the absence or presence of activated MMP-9 (0.8–500 nM), cd-MMP-3 (3.3 nM and 100 nM), MMP-1, -2, -8, -13 (5 nM and 100 nM), 100 mM EDTA and 400 μM Ac-DEVD-CHO (*N*-Acetyl-Asp-Glu-Val-Asp-aldehyde, Sigma).

Digestion of recombinant CAP1 by activated MMP-9 variants

Recombinant glutathione-*S*-transferase (GST)-tagged CAP1 (Abnova, Niehu, Taipei, Taiwan) was dialyzed against assay buffer to remove residual glutathione. The protein concentration was determined with the Bradford protein assay (Bio-Rad, Hercules, CA, USA) as described [11] with bovine serum albumin (Sigma) as a standard. 315 ng CAP1 was incubated during 75 min in the absence or presence of the activated MMP-9 variants at the indicated concentrations (0.37–29 nM): full-length MMP-9, MMP-9 with deletion of the *O*-glycosylated domain (MMP-9ΔOG), of the hemopexin domain (MMP-9ΔHem), and with deletion of both the hemopexin and *O*-glycosylated domains (MMP-9ΔOGHem) [10]. As a control, all MMP-9 variants were also incubated separately in assay buffer.

SDS-PAGE and Western blot analysis

Cell extracts, (digested) culture media of THP-1 cells and recombinant CAP1 digestions were analyzed by reducing Tris-glycine SDS-PAGE (9% and 15% acrylamide) and visualized by silver staining analysis (Silverquest™ Silver Staining kit, Invitrogen). For Western blot analysis, THP-1 cytosol digestions, prepared as described above, were separated by reducing Tris-tricine SDS-PAGE (14% Prosieve® 50 Gel Solution, Lonza, Basel, Switzerland). After electrophoresis, the proteins were transferred from the gels to PVDF (Millipore Corporation, Billerica, MA, USA) or nitrocellulose membranes (0.2 μm, Sigma) by electroblotting. Blotting was performed semi-dry for all blots, except for the blots of the urine samples (*vide infra*), which were wet-blotted. The membranes were blocked with 5% non-fat dry milk (Bio-Rad) in Tris-buffered saline (20 mM Tris/HCl, pH 7.6, 137 mM NaCl) containing 0.1% Tween-20 and then probed with a mouse polyclonal antibody raised against full-length recombinant GST-tagged CAP1 (Abnova). After washing, the membrane was incubated with a peroxidase-conjugated secondary goat anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA, USA) and after the final wash, proteins were detected according to the manufacturer's recommendations of the ECL plus™ chemiluminescence kit (GE Healthcare, Uppsala, Sweden). Alternatively, the IgG fraction of the polyclonal CAP1 antiserum was biotinylated after purification on a GammaBind Plus Sepharose column (GE Healthcare) and peroxidase-conjugated streptavidin (Jackson ImmunoResearch) was used as secondary reagent. For fluorescent detection, the purified polyclonal CAP1 antibody was labeled with Cy5-NHS (GE Healthcare) resulting in a final dye/product (D/P) ratio of 6.4/1. The monoclonal CAP1 antibody (Abnova) was labeled with Cy3-NHS (GE Healthcare), giving a final D/P ratio of 4.8/1. Non-immune IgG was purified from murine serum (C57Bl/6) on a GammaBind Plus Sepharose column and subsequently labeled with Cy3-NHS, resulting in a final D/P ratio of 6.2/1. Urine blots were probed with the Cy5-conjugated polyclonal CAP1 antibody (4 μg/ml) and the Cy3-labeled non-immune IgG (4 μg/ml), followed by probing with Cy3-labeled monoclonal CAP1 antibody (2 μg/ml) and fluorescence was detected on the Ettan DIGE Imager (GE Healthcare).

NH_2-terminal Edman sequencing and protein identification by LC-MS/MS

Proteins were separated by SDS-PAGE and electroblotted onto PVDF membranes. After washing with H_2O and staining with Coomassie Brilliant Blue R-250 (Sigma), the protein band(s) of interest were excised from the blot and applied to a capillary protein sequencer (Procise 491 cLC, Applied Biosystems, Foster City, CA, USA) for NH_2-terminal Edman degradation. For LC-MS/MS, proteins were stained with Coomassie Brilliant Blue R-250 after SDS-PAGE analysis and the protein band(s) of interest were excised from the gel and identified by electrospray ionization (ESI)-tandem mass spectrometry (MS/MS) after in-gel tryptic digest (Eurogentec S.A., Seraing, Belgium).

Patients and urine samples

All patients contributing to the present study gave informed consent and the study protocol was in accordance with international standards and approved by the Local Ethical Committee for clinical studies. Clinical and laboratory data were decoded and provided by the treating physician-specialist. Urine samples from the patients and four healthy volunteers were collected on ice and immediately (<15 min) frozen at −78.5 °C on dry ice before analysis. For zymographic analysis, 0.5 ml urine was first prepurified on gelatin-Sepharose as described [12]. Gelatin zymography was performed as previously detailed [9] and the relative migrations of pro-gelatinase B in covalent complex with neutrophil gelatinase B-associated lipocalin (NGAL), of the pro-MMP-9 and pro-MMP-2, as well as of the activated forms thereof, were determined with the use of laboratory standards. Western blot analysis of CAP1 in 50 μl urine per sample was carried out with Cy dye-labeled primary antibodies and fluorescent detection, as described above.

Results

Gelatinase B/MMP-9 clears intracellular proteins from dying THP-1 cells

In order to study the accessibility of intracellular proteins towards extracellular proteases, an experimental model of cell death by nutrient deprivation was used in a cell line of the myeloid lineage. Human myelomonocytic THP-1 cells were incubated in serum-free conditions for 40 h. To prevent released proteins from degradation, a general protease inhibitor cocktail (PIn) was added, as well as 1,10-*o*-phenanthroline (O-Phen). The pan-caspase inhibitor Z-VAD. fluoromethylketone (Z-VAD.fmk) was used to counter apoptosis induction. The percentages of apoptotic and necrotic cells were assessed by flow cytometry (Fig. 1) as described in the section 'Materials and methods'. In comparison with untreated cells, the addition of PIn and O-Phen induced additional cytolysis, which was diminished by Z-Vad.fmk. As expected from the flow cytometry data, considerable cytolysis was obvious after 40 h, resulting in abundant protein content in the culture media. To

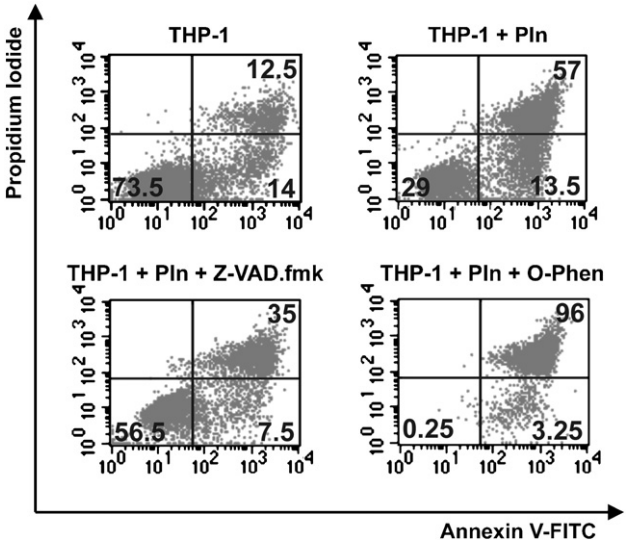

Fig. 1 – Incubation of THP-1 cells in serum-free medium induces cytolysis. THP-1 cells were incubated in serum-free medium for 40 h to induce cell death by nutrient deprivation. To prevent released proteins from being degraded, a general protease inhibitor cocktail (PIn) was added, as well as 1,10-*o*-phenanthroline (O-Phen). The pan-caspase inhibitor Z-Val-Ala-Asp.fluoromethylketone (Z-VAD.fmk) was used to counter apoptosis induction. Annexin-V-positive cells represent the early apoptotic cells (lower right quadrants), whereas the double-positive cells correspond to late apoptotic or necrotic cells (upper right quadrants). The residual viable cells are double-negative (lower left quadrants). The numbers within each quadrant indicate the cell percentages within the total cell populations.

avoid the aspecific cytolysis caused by PIn and O-Phen, shorter incubation times (<5 h) were combined with the use of high cell densities (10×10^6 cells/ml) as this combines the benefit of high levels of released THP-1 proteins in the culture media with a shorter incubation of these proteins in the presence of aggressive cellular proteases. When these culture media were compared with THP-1 cell extracts by SDS-PAGE analysis, most proteins in the culture media corresponded to extracted cellular proteins (Fig. 2A).

In order to define MMP-9 substrates, THP-1 cells were incubated in the absence or presence of recombinant human MMP-9, activated with the catalytic domain of stromelysin-1/MMP-3 (cd-MMP-3). Subsequently, equivalent amounts of culture media were analyzed by SDS-PAGE. After incubation of dying THP-1 cells for 4.5 h in the presence of activated MMP-9 (1 μM), one major and several minor protein bands disappeared, whereas other low-abundancy (fragment) bands became visible (Fig. 2B). Control incubations with equivalent amounts of cd-MMP-3 (10 nM), used to activate pro-MMP-9, did not show any protein degradation, ruling out that cd-MMP-3 would be the effector of the protein degradation. To distinguish cellular protein bands from MMP-9 bands, an equivalent amount of MMP-9 (1 μM) was incubated in serum-free medium and analyzed in parallel. The detection of several differential bands confirms that various THP-1 proteins are efficiently fragmented or cleared by MMP-9.

An abundant 57 kDa protein is efficiently cleaved by MMP-9

Most prominently, an abundant 57 kDa protein band completely disappeared after 4.5 h incubation in the presence of MMP-9 (closed arrowhead, Fig. 2B). In order to examine the cleavage efficiency of this 57 kDa protein, culture media of THP-1 cells were incubated in the presence of various concentrations of activated MMP-9. The 57 kDa protein was proteolysed in an MMP-9 concentration-dependent way (Fig. 3). Complete turnover of the 57 kDa substrate was achieved with only 9 nM MMP-9 and partial cleavage still occurred at 0.36 nM MMP-9. This corresponded to a cleavage efficiency comparable with that of gelatin, the most efficient MMP-9 substrate [10].

Inhibition profiling confirms cleavage of the 57 kDa protein by MMP-9

In order to verify that proteolysis of the 57 kDa protein indeed results from MMP-9 action, inhibition tests with various protease inhibitors were performed. Protein profiling of THP-1 culture media, containing the 57 kDa protein, with 20 nM MMP-9 in the presence or absence of inhibitors, showed that cleavage of the 57 kDa protein was MMP-specific. In the presence of EDTA (a general metalloproteinase inhibitor) and TIMP-1 (which inhibits mainly MMPs and MMP-9 with a particularly high affinity) the

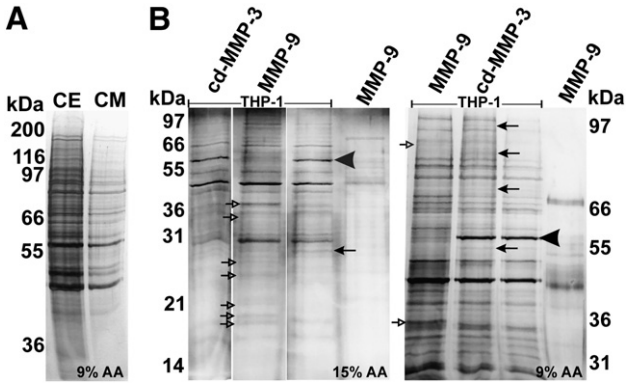

Fig. 2 – MMP-9 clears intracellular proteins released by dying THP-1 cells. (A) THP-1 cells were incubated in serum-free conditions during 4.5 h. After separation by centrifugation, cell extract (CE) and culture medium (CM) were analyzed by SDS-PAGE. Many protein bands in the culture medium match in migration profile with cell extract proteins, indicating that cell death leads to the release of many intracellular THP-1 proteins into the culture medium. (B) THP-1 cells were incubated during 4.5 h in the absence or presence of MMP-9 (1 μM). As a control, MMP-9 (1 μM) was incubated in serum-free medium without THP-1 cells. In the culture media of THP-1 cells incubated in the presence of activated MMP-9, some protein bands disappeared (closed arrows and arrowhead), while various protein fragments became visible (open arrows). Negative control incubation with cd-MMP-3 (10 nM) (used to activate pro-MMP-9) did not result in obvious protein degradation. The acrylamide (AA) gel percentages used to separate the proteins are shown at the right bottom of each gel panel. A representative protein profile of 4 independent experiments is shown.

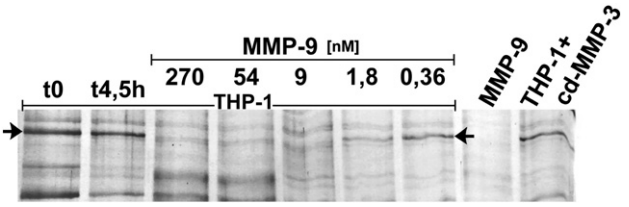

Fig. 3 – An abundant 57 kDa protein is cleaved with high efficiency. Culture medium of THP-1 cells containing the 57 kDa protein (arrows) was incubated for 4.5 h in the absence or presence of various MMP-9 concentrations. MMP-9 (270 nM) incubated in serum-free medium was used as a control. Cleavage of the 57 kDa protein was observed with MMP-9 concentrations as low as 0.36 nM. Control incubations with 2.7 nM cd-MMP-3 did not cause degradation of the 57 kDa protein band. Analysis of culture medium of THP-1 cells before incubation with MMP-9 is marked as t0. THP-1 culture medium after 4.5 h of incubation without MMP-9 is indicated by t4.5h. A representative protein profile of 2 independent experiments is shown.

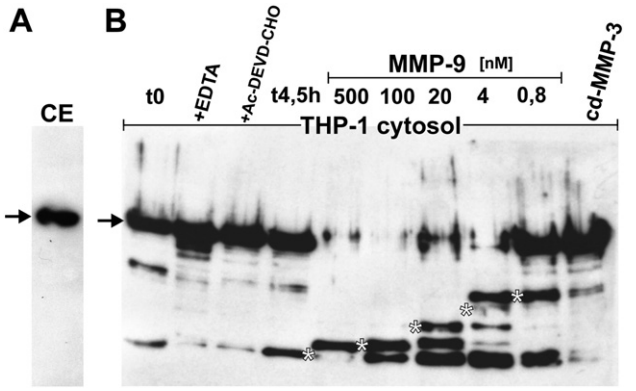

Fig. 5 – Western blot analysis confirms efficient CAP1 cleavage by MMP-9. (A) Specificity of the CAP1 antibody was confirmed with THP-1 cell extract (CE), showing a single protein band at 57 kDa (arrow). (B) Initial CAP1 fragments, present in the cytosol extract before incubation, are shown in the first lane (t0). Control incubations included addition of EDTA and the caspase-3/7 inhibitor Ac-DEVD-CHO. THP-1 cytosol was incubated for 4.5 h without MMP-9 (t4.5h) or in the presence of various MMP-9 concentrations. All incubations were subjected to Western blot analysis with a polyclonal antibody against recombinant CAP1, showing efficient degradation of the CAP1 band (arrow) by MMP-9. Multiple fragment bands appear (*) and are further degraded at higher MMP-9 concentrations. Addition of equivalent amounts of cd-MMP-3 (3.3 nM) did not result in CAP1 fragmentation.

substrate remained intact when MMP-9 was added (Supplementary Fig. 1). However, addition of the serine protease inhibitors Pefabloc® and benzamidine, or the thiol protease inhibitor E64, did not prevent degradation, confirming that MMP-9 is responsible for this cleavage. The fact that released cellular proteases also present in the culture medium do not degrade the 57 kDa protein (Supplementary Fig. 1, t4.5h *vs.* t0) is an additional indication of the high specificity of this cleavage.

Identification of CAP1 as the 57 kDa MMP-9 substrate by LC-MS/MS

Culture medium of THP-1 cells was incubated in the absence or presence of MMP-9, and the 57 kDa protein was subjected to NH_2-terminal Edman sequencing as described in the section 'Materials and methods'. However, the 57 kDa protein appeared to be resistant to Edman degradation. Hence, the 57 kDa protein was submitted to LC-MS/MS analysis after in-gel tryptic digest and identified as adenylyl cyclase-associated protein-1 (GenBank accession no. AAP35816) or CAP1 (Fig. 4), an intracellular protein with a theoretical mass of 51,641 Da, which is involved in actin polymerization [13].

Western blot analysis of CAP1 proteolysis

In order to confirm the LC-MS/MS identification of CAP1 as an MMP-9 substrate, recombinant GST-tagged CAP1 (315 ng) was incubated with various concentrations of activated MMP-9. Recombinant CAP1 was cleaved by MMP-9 concentrations as low as 0.37 nM and complete degradation was observed with 3.2 nM, which corresponded to enzyme:substrate ratios of 1:1175 and 1:145, respectively (Supplementary Fig. 2). Densitometric analysis of the intact recombinant CAP1 band after incubation in the absence or presence of MMP-9 yielded a CC_{50} (enzyme concentration resulting in 50% cleavage) of 0.5 nM after only 75 min of incubation. This pointed again to a highly efficient proteolysis, as the cleavage of the prototypic MMP-9 substrate, gelatin, is

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  1 MADMQNLVER LERAVGRLEA VSHTSDMHRG YADSPSKAGA APYVQAFDSL LAGPVAEYLK
 61 ISKEIGGDVQ KHAEMVHTGL KLERALLVTA SQCQQPAENK LSDLLAPISE QIKEVITFRE
121 KNRGSKLFNH LSAVSESIQA LGWVAMAPKP GPYVKEMNDA AMFYTNRVLK EYKDVDKKHV
181 DWVKAYLSIW TELQAYIKEF HTTGLAWSKT GPVAKELSGL PSGPSAGSCP PPPPPCPPPP
241 PVSTISCSYE SASRSSLFAQ INQGESITHA LKHVSDDMKT HKNPALKAQS GPVRSGPKPF
301 SAPKPQTSPS PKRATKKEPA VLELEGKKWR VENQENVSNL VIEDTELKQV AYIYKCVNTT
361 LQIKGKINSI TVDNCKKLGL VFDDVVGIVE IINSKDVKVQ VMGKVPTISI NKTDGCHAYL
421 SKNSLDCEIV SAKSSEMNVL IPTEGGDFNE FPVPEQFKTL WNGQKLVTTV TEIAG
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Fig. 4 – Identification of CAP1 as a prominent MMP-9 substrate. Culture medium of THP-1 cells was incubated in the absence or presence of MMP-9 and separated by SDS-PAGE, followed by staining with Coomassie Blue. The 57 kDa protein band was excised and subjected to LC-MS/MS analysis after in-gel tryptic digest. It was identified as adenylyl cyclase-associated protein-1 or CAP1. LC-MS/MS peptides matching with the CAP1 sequence are shown on a black background. The central poly-proline stretch (residues 230–241) is in italics and boxed in a gray background.

characterized by a CC_{50} of 1 nM after 60 min [10]. As CAP1 is an intracellular protein, the extracted cytosolic fraction of THP-1 cells was used as a natural source of CAP1 to further confirm the identification and cleavage by Western blot analysis. THP-1 cytosol incubated during 4.5 h in the absence or presence of MMP-9 was subjected to Western blot analysis with a polyclonal antibody against recombinant CAP1. Specificity of the CAP1 antibody was confirmed with THP-1 cell extract, showing only one protein band at 57 kDa (Fig. 5A). The procedure of cytosol extraction was the cause of the initial fragmentation seen before incubation (Fig. 5B). Control incubations in the absence of MMP-9 included addition of EDTA, the caspase-3/7 inhibitor Ac-DEVD-CHO, and equivalent amounts of cd-MMP-3. The protective effect observed with EDTA shows that CAP1 might also be degraded by an endogenous metalloprotease. This enzyme may be endogenous MMP-9, as well as another endogenous MMP or another metalloproteinase, as the inhibitory power of EDTA extends to all the proteases that depend on light metal cations for activity. The dose-dependent fragmentation pattern generated by MMP-9 confirms the specificity and efficiency of CAP1 degradation by MMP-9.

The kinetics of CAP1 cleavage were investigated by incubating THP-1 cytosol with 4 nM activated MMP-9 and visualization of cleavage at different time points by Western blot analysis (Fig. 6A). Semi-quantitative kinetic data were obtained from densitometric scanning of the intact CAP1 band (arrow, Fig. 6B). After 1.5 h, 60% of CAP1 was cleared, leaving less than 20% of the intact substrate after 4.5 h of MMP-9 action. In summary, a fast and highly efficient clearance of CAP1 by MMP-9 was corroborated by Western blot analysis.

CAP1 cleavage is confirmed with MMP-9 deletion mutants

Binding of MMP substrates to enzyme exosites, which are discrete substrate-binding sites on domains outside the catalytic domain, enhances considerably the substrate affinity and is crucial for efficient proteolysis of particular substrates [14]. For example, the hemopexin domain is indispensable for classical collagenases (MMP-1, -8 and -13) to cleave native, triple helical collagens, since deletion of the hemopexin domain results in loss of their collagenolytic capacity [15]. As the CAP1 cleavage efficiency was in the same order of magnitude as gelatin degradation, binding of CAP1 to an MMP-9 exosite might be involved. In order to investigate potential roles of the *O*-glycosylated domain and the hemopexin domain of MMP-9 in the cleavage of CAP1, recombinant CAP1 was incubated with full-length recombinant MMP-9, as well as with three recombinant MMP-9 domain deletion mutants. More specifically, we used MMP-9 variants with deletion of the hemopexin domain (MMP-9ΔHem), the *O*-glycosylated domain (MMP-9ΔOG) and with deletion of both the hemopexin and *O*-glycosylated domains (MMP-9ΔOGHem). As can be observed from Supplementary Fig. 2, CAP1 was efficiently cleaved by the wildtype and by the three domain deletion mutants. No difference in cleavage efficiency was observed between the MMP-9 variants, demonstrating that neither the hemopexin domain, nor the *O*-glycosylated domain played a key role in CAP1 cleavage.

CAP1 degradation is an MMP-9-specific process

Frequently, MMP substrates can be cleaved by different MMPs according to the pathophysiological context [1,3]. In order to investigate whether CAP1 degradation might also be performed by other MMPs, THP-1 cytosol was incubated for 4.5 h with 5 and 100 nM of the gelatinases MMP-9 and MMP-2, of the collagenases MMP-1, -8 and -13, as well as with 100 nM of the catalytical domain of stromelysin-1 (cd-MMP-3). Western blot analysis with the biotinylated polyclonal CAP1 antibody showed that at high MMP concentrations (100 nM) total clearance of the intact CAP1 band, indicated by the arrow, was achieved by MMP-9, -2, -8 and -13, whereas MMP-1 was hardly capable of fragmentation and cd-MMP-3 did not cleave CAP1 at all (Fig. 7A). Thus, extracellular CAP1 cleavage was achieved by different MMPs. However, CAP1 degradation by MMP-9 was more pronounced as it generated the smallest fragments. In addition, at lower protease concentrations (5 nM), MMP-9 was the only MMP that was still capable of CAP1

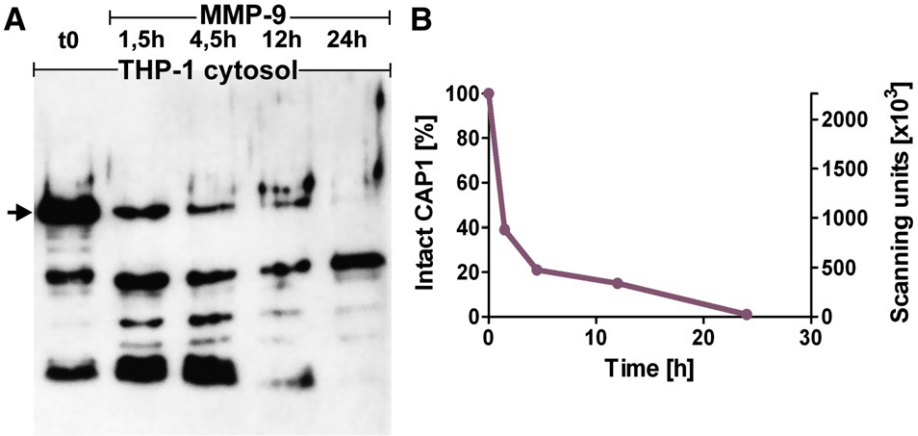

Fig. 6 – Semi-quantitative kinetics of CAP1 cleavage by MMP-9. (A) THP-1 cytosol was incubated in the presence of 4 nM activated MMP-9. Samples were taken at the indicated time points and analyzed by Western blotting with a polyclonal CAP1 antibody for detection. Densitometric analysis of the intact CAP1 band (arrow) yielded semi-quantitative kinetic data, which were plotted in a graph *vs.* duration of incubation. Analysis of THP-1 cytosol before incubation is marked as t0. (B) 60% of CAP1 was degraded after 1.5 h of incubation and an additional 20% was cleared during the next 3 h. Fragments, present at the start or generated by MMP-9 after 1.5 h, were further degraded after 12 to 24 h of incubation.

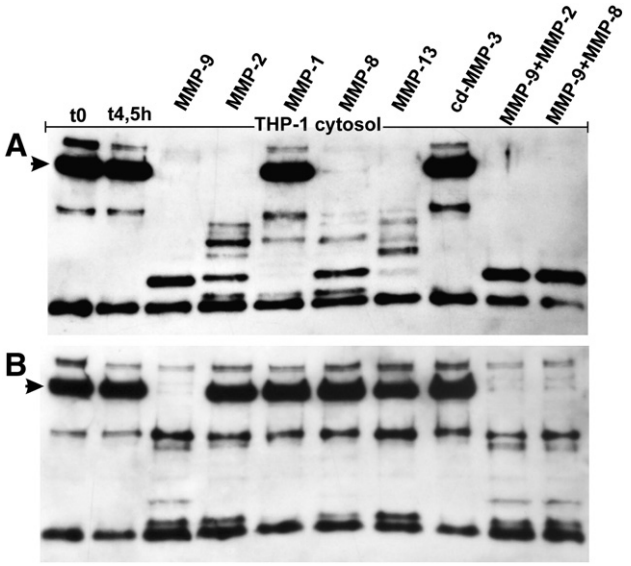

Fig. 7 – CAP1 degradation is an MMP-9-specific process. THP-1 cytosol was incubated for 4.5 h without MMP (t4.5h) or in the presence of 100 nM (A) and 5 nM (B) of activated MMP-9, -2, -1, -8, -13 and the catalytic domain of MMP-3 (cd-MMP-3). Synergistic actions between MMP-9 and MMP-2 or MMP-8 were investigated by co-incubation of both MMPs with THP-1 cytosol for 4.5 h. All incubations were analyzed by SDS-PAGE and Western blot analysis with a biotinylated polyclonal CAP1 antibody. A. At high protease concentrations total degradation of the intact CAP1 band (arrow) is achieved by MMP-9, -2, -8 and -13, whereas little or no cleavage is observed with MMP-1 and cd-MMP-3, respectively. B. In the presence of physiologic MMP concentrations, only MMP-9 is still capable of CAP1 degradation, showing that MMP-9 is most efficient at CAP1 clearance. Analysis of THP-1 cytosol before incubation is marked as t0.

clearance (Fig. 7B), showing that – of the MMPs tested – MMP-9 was most efficient at CAP1 degradation. In order to investigate synergistic actions between MMP-9 and MMP-2 or MMP-8, THP-1 cytosol was co-incubated for 4.5 h with 5 and 100 nM of MMP-9 and MMP-2 or -8. However, at both concentrations the fragmentation patterns were identical to those generated by MMP-9 alone, suggesting that in the presence of MMP-9, less efficient MMPs did not contribute to better CAP1 cleavage. In conclusion, CAP1 was efficiently cleaved by 5 nM MMP-9, a concentration that is physiologically attainable in many body compartments.

Analysis of coincident CAP1 and MMP-9 in vivo

To substantiate the *in vivo* relevance of the previous biological and biochemical findings, we undertook additional studies of clinical samples from leukemia patients under chemotherapeutic treatment, and from patients with systemic autoimmune diseases. We first screened a limited number of plasma and serum samples from such patients by the described Western blot technique. However, direct detection of CAP1 in plasma and serum samples was not possible because of the overlapping molecular weights of CAP1 (57 kDa) and the highly abundant serum proteins albumin (migrating between 50 and 75 kDa on SDS-PAGE) and the immunoglobulin heavy chain (50–55 kDa on SDS-PAGE), which mask the CAP1 band. In view of our anticipation that intact CAP1 (57 kDa) and consequently also CAP1 fragments might pass through the kidney filter, we examined available urine samples from patients with systemic autoimmune diseases. In addition, CAP1 was previously identified as an autoantigen in a systemic lupus erythematosus (SLE) patient with active disease and nephritis [16] and in rheumatoid arthritis (RA) patients [17]. Other types of autoimmune diseases, including Sjögren's syndrome (SS) and arteritis temporalis (AT) are as well characterized by tissue damage and vascular involvement that may lead to circulating CAP1. Relevant clinical information of 11 patients with SLE, SS or AT was collected (Table 1). An increased C-reactive protein (CRP) level reflects an acute inflammation, whereas increased serum creatinine levels and proteinuria are indicative for renal failure.

The primary mouse polyclonal CAP1 antibody was labeled with the fluorescent dye Cy5 for direct fluorescence detection of immunoreactivity (Fig. 8, panel A). Intact CAP1 was detected in the urines of patients and controls, except for patients P10 and P11. Interestingly, CAP1 was clearly increased in the urine of some patients (e.g. P1, P3, P5 and P6) in comparison with control urine samples (C1–C4). In a few patients, immunoreactive bands at a lower molecular weight were detected (* and open arrowheads) that were potential CAP1 fragments. To investigate the specificity

Table 1 – Diagnosis and clinical parameters of patients with systemic autoimmune diseases

	Diagnosis	Sex (M/F)	Age (years)	CRP (mg/l)	Serum creatinine (mg/dl)	Increased proteinuria
P1	SLE (drug-induced)	F	15	2.4	0.71	No
P2	SLE	M	71	2.1	***1.54***[a]	ND
P3	SLE	F	37	7.8	0.97	ND
P4	SLE	F	79	<1	0.79	No
P5	SLE	F	77	<1	***1.36***[a]	***Yes***
P6	SLE	F	21	<1	0.91	***Yes***
P7	SLE	F	27	1.2	***1.07***[a]	***Yes***
P8	AT	F	73	1	0.95	***Yes***
P9	SS	F	54	1	0.92	ND
P10	SS	F	40	***26***[a]	***1.7***[a]	ND
P11	SS and cryoglobulinemia	F	71	3	***1.06***[a]	***Yes***

SLE, systemic lupus erythematosus; AT, arteritis temporalis; SS, Sjögren's syndrome; M, male; F, female; CRP, C reactive protein.
[a] Values in bold and italics are above the healthy threshold for the measured parameter.

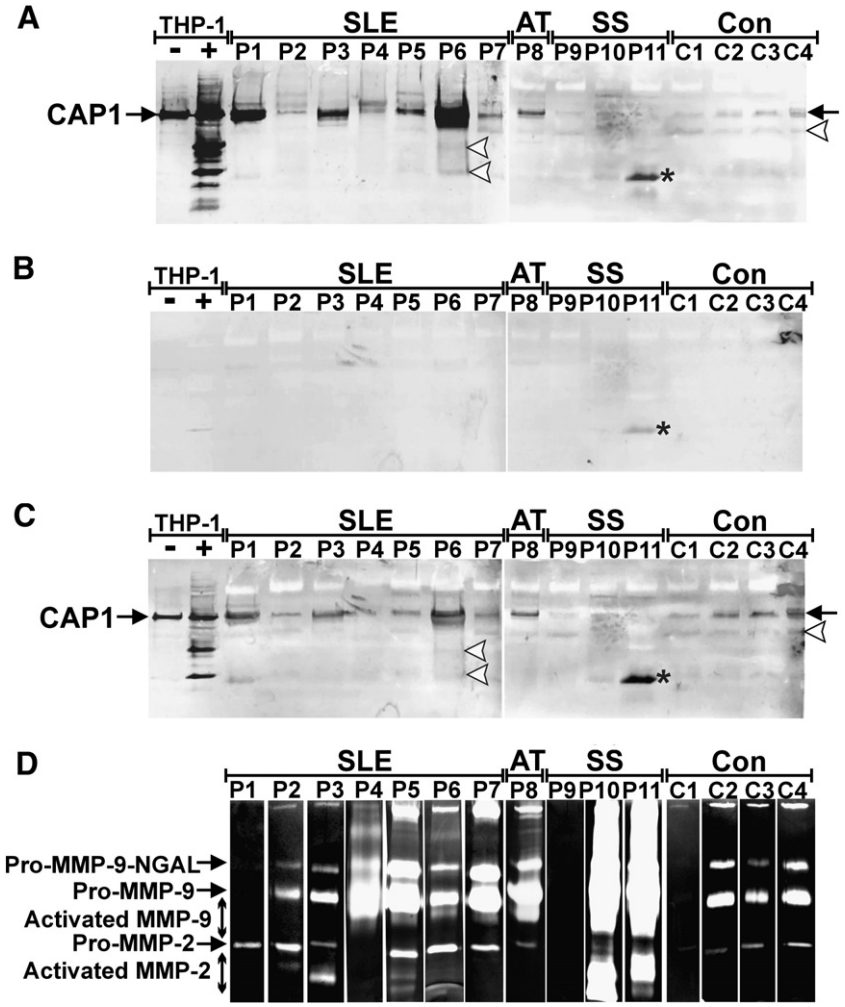

Fig. 8 – Coincident CAP1 and MMP-9 in urine samples of patients with systemic autoimmune diseases. Urine samples (50 μl) from 11 patients (P1–P11) with systemic lupus erythematosus (SLE), arteritis temporalis (AT) or Sjögren's syndrome (SS) and 4 healthy controls (Con) were subjected to Western blot analysis. THP-1 cytosol incubated for 4.5 h with EDTA (first lane, THP-1, −) was used as a source of intact CAP1 (closed arrows) and was spiked into 8-fold amounts of THP-1 cytosol incubated during 4.5 h with 4 nM MMP-9 as a positive control for MMP-9-generated CAP1 fragments (second lane, THP-1, +). (A) Blotted urine samples were analyzed with a Cy5-labeled polyclonal CAP1 antibody (final concentration 4 μg/ml). Intact CAP1 (closed arrows) was detected in the urines of healthy controls and in patient urines, except for P10 and P11. In some patients, immunoreactive bands were detected at lower molecular weight (* and open arrowheads). (B) To confirm the specificity of the immunoreactive bands, the same urine blots were also probed with non-immune IgG, labeled with Cy3 (final concentration 4 μg/ml). Hence, one potential fragment band was shown to be non-specific (*). (C) As a final validation of CAP1 detection *in vivo*, the blots were then probed with Cy3-conjugated monoclonal CAP1 antibody (final concentration 2 μg/ml), showing similar detection of intact CAP1 (closed arrows) and CAP1 fragments (open arrowheads) as with the polyclonal antibody in panel A. (D) Urine samples from patients and controls (500 μl) were prepurified on gelatin-Sepharose beads and analyzed by gelatin zymography. The highest MMP-9/gelatinase B and MMP-2/gelatinase A levels were found in urines samples from P10 and P11 with SS and indications of renal injury. Whereas no activated MMP-9 was detected in control urines, all urines of patients with pathological clinical parameters suggesting renal failure contained activated MMP-9. Pro-MMP-9-NGAL is neutrophil MMP-9 complexed with gelatinase B-associated lipocalin (NGAL).

of the immunoreactive bands, non-immune IgG was purified from murine serum and labeled with Cy3 under the same conditions used to purify and label the polyclonal CAP1 antibody. Since fluorescent detection on Western blots is suitable for multiple labeling, the same urine blots were then probed with the Cy3-labeled non-immune murine IgG, which showed the specificity of the intact CAP1 band, as it was not detected with non-immune IgG (Fig. 8, panel B). However, non-immune IgG reacted with one of the potential CAP1 fragments (*), showing that this was a non-specific immunoreactivity. In contrast, the other immunoreactive bands at lower molecular weight (open arrowheads) were not observed with non-immune IgG. As an additional confirmation of CAP1 detection *in vivo*, the same urine blots were subsequently probed with a Cy3-conjugated monoclonal CAP1 antibody (Fig. 8, panel C).

Intact CAP1 and CAP1 fragments (open arrowheads) reappeared and were indeed recognized by both the polyclonal and monoclonal antibodies.

For the detection of the CAP1-degrading enzyme MMP-9, 500 μl of the urine samples was prepurified on gelatin-Sepharose, as previously described [12]. When urinary gelatinase A/MMP-2 and gelatinase B/MMP-9 levels were analyzed by zymography (Fig. 8, panel D), the highest levels were found in urines from patients P10 and P11 with SS and indications of renal damage. To facilitate the interpretation of the P10 and P11 zymograms, the equivalent of only 20 μl of urine after prepurification was analyzed and shown in parallel in Supplementary Fig. 3. Interestingly, all patients with clinical parameters suggesting renal damage had activated MMP-9 and/or MMP-2 in their urines. In the urines of patients P10 and P11 with the highest level of pro- and activated MMP-9 and MMP-2, the intact CAP1 band was decreased in comparison with the CAP1 levels in the urines of healthy control donors. In addition, in patients with a clear but less pronounced increase of pro- and activated MMP-9 (P4, P5, P7 and P8), CAP1 levels were slightly higher than in the controls, whereas in samples without MMP-9 or with low levels of activated MMP-9, an increased amount of CAP1 was detected (P1, P3 and P6). These inverse relations between the levels of intact CAP1 and activated MMP-9, as well as the detection of possible CAP1 fragments, suggest that MMP-9 is involved in CAP1 degradation *in vivo*.

In conclusion, we demonstrated the presence of intact extracellular CAP1 in the urine of patients with systemic autoimmune diseases and healthy controls. Whereas healthy control urines did not contain active MMP-9, all urine samples of patients with clinical parameters pointing to renal failure showed activation of MMP-9 and/or MMP-2. In addition, in some patient urines with an overload of pro- and activated MMP-9 and/or MMP-2, the intact CAP1 band disappeared, whereas urines from patients without urinary MMP-9 or with low levels of active MMP-9 contained an increased level of intact CAP1.

Discussion

MMPs in general, and MMP-9 in particular, cleave extracellular structural (e.g. gelatins) and functional substrates (e.g. chemokines). Proteolysis by gelatinase B/MMP-9 is of physiological importance in reproduction and development, leukocyte mobilization and tissue repair. MMP-9 is known for its contribution to the pathogenesis of inflammation, vascular diseases, cancer and autoimmune diseases [18]. Indeed, MMP-9 was proposed to act as a switch and catalyst in both innate and specific immunity as it activates pro-inflammatory cytokines such as IL-1β, modifies chemokine activities and assists in leukocyte mobilization and migration across basement membranes. MMPs cleave autoantigenic molecules into immunodominant epitopes for T cells [19]. However, the knowledge of intracellular MMP substrates is rather limited.

In this study, a myeloid cell model system was used to define critical and efficient MMP-9 substrates associated with cytolysis. CAP1 was thus identified by mass spectrometry analysis and the cleavage efficiency was corroborated by Western blot analysis. Recombinant CAP1 was degraded at enzyme:substrate ratios exceeding 1:1000 and at an estimated CC_{50} of 0.5 nM after 75 min of incubation. Hence, CAP1 degradation is more efficient than cleavage of gelatin (CC_{50} of 1 nM after 60 min incubation) and βB1 crystallin (CC_{50} of 3 nM after 180 min incubation) [7,10], the two most efficient known MMP-9 substrates. As exosite interactions may be crucial for a high cleavage efficiency [14], it was hypothesized that CAP1 would bind to an MMP-9 exosite. However, with the use of MMP-9 domain deletion mutants, the hemopexin domain and the *O*-glycosylated domain were excluded as modules bearing exosites for CAP1 binding. The fibronectin domain of gelatinases is required for the binding of gelatin, laminin and collagens type I and IV. In addition, binding to exosites in the fibronectin domain strongly enhances gelatin affinity [20]. Interestingly, CAP1 contains a centrally located stretch of poly-proline residues (*cfr.* Fig. 4) [21] and may possibly mimic exosite binding of proline-rich gelatin to the fibronectin domain, in this way acquiring the ideal position for efficient cleavage. However, by immunoprecipitation experiments, interactions between CAP1 and the MMP-9 fibronectin domain, or any other MMP-9 exosite, were ruled out (data not shown), which leads us to the conclusion that the interaction with the catalytic site of MMP-9 is ideal and leads to the high cleavage rate of CAP1. In addition, when comparing CAP1 degradation by various MMPs, we found that MMP-9 was the most efficient CAP1-degrading MMP and the only protease of the tested MMPs to clear CAP1 at low protease concentrations. This showed that CAP1 degradation by MMP-9 is not only a highly efficient but also an MMP-specific process.

Cyclase-associated proteins or CAPs constitute a family of highly conserved monomeric actin-binding proteins (molecular weight: 50–60 kDa) present in a wide range of organisms including yeasts, flies, plants and mammals. CAP (Srv2/ASP56) was originally isolated as a component of the *Saccharomyces cerevisiae* adenylyl cyclase complex, which is implicated in Ras signaling. The C-terminal-binding site for monomeric or globular (G)-actin is well conserved in CAP1 molecules in various species [13]. In addition, all CAP homologs contain a centrally located proline-rich region, which is recognized by the SH3-domains of several proteins, suggesting that CAP could mediate interactions between SH3-domain proteins and monomeric actin [21]. In mammals two different CAP genes have been found, CAP1 and CAP2, which share 64% amino acid identity [22,23]. Analysis of CAP1-deficient cells showed that it promotes rapid actin dynamics and is important for cell morphology, migration and endocytosis, as well as for the localization and function of actin-depolymerizing cofilin [24,25].

Several reasons incited us to use THP-1 cells as an experimental cell model. One rationale to use and to induce apoptosis/necrosis in this human myelomonocytic leukemia cell line THP-1 was drawn from the knowledge that chemotherapy for leukemia induces cytolysis. Moreover, in tumor lysis syndrome, aggressive chemotherapy causes rapid tumor cell destruction and a massive release of intracellular contents into the systemic circulation. This results in metabolic disturbances, leading to multi-organ-failure and even death [26]. The highly efficient clearance of CAP1 by MMP-9 may be important during such (extensive) cytolysis. Indeed, dying cells release intracellular proteins into the extracellular milieu, creating a strong pro-inflammatory stimulus which engenders or enhances inflammation. The intracellular material can be removed by phagocytes or first be degraded by proteases, such as MMP-9, which is stored in the granules of neutrophils and released within minutes to 1 h after neutrophil stimulation [9]. In addition, CAP1 was identified as an

endothelial cell-associated autoantigen in a systemic lupus erythematosus patient with active disease and nephritis [16] and in rheumatoid arthritis patients [17]. As CAP1 is an abundant and ubiquitous protein, the efficient clearance of this antigen may be of physiological importance to avoid autoimmune or other reactions.

Another potential effect of fast CAP1 degradation may be the removal of the actin monomer-sequestering activity of CAP1. Indeed, when cells die, monomeric and filamentous actins are released into the extracellular space and reach the systemic circulation. In the plasma, where the ionic strength, pH and temperature promote polymerization, actin monomers can form long filaments together with coagulation factor Va, which triggers disseminated intravascular coagulation if not rapidly resolved [27–30].

Interestingly, other actin-binding proteins have also been described as MMP substrates. α-Actinin, which is known to connect actin filaments of adjacent sarcomeres, is cleaved by MMP-2 *in vitro* and it is hypothesized that this proteolysis would mediate cytoskeletal disorganization and peroxynitrite-induced injury in the heart [31]. Gelsolin is cut into several fragments by various MMPs, including MMP-9, resulting in considerable loss of its actin-depolymerizing activity [32,33], which may as well lead to intravascular coagulation by increasing the concentration of actin filaments in the circulation.

In conclusion, CAP1 is released upon cytolysis into the extracellular milieu *in vitro* and thus becomes a highly efficient MMP-9 substrate. In addition, we showed that the CAP1 antigen can be detected by Western blot analysis in only 50 μl of urine without prepurification steps. Hence, CAP1 is present in the extracellular milieu *in vivo*. In some urine samples of patients with systemic autoimmune diseases, an inverse relation was observed between the levels of intact CAP1 and activated MMP-9/-2, suggesting CAP1 degradation by MMP-9/-2 in these patients. Degradation of intracellular proteins may result in the generation of new autoantigens [34,35]. In addition, CAP1 degradation by MMP-9/-2 might affect acute pathological conditions caused by extracellular actin toxicity. Therefore, further elucidation of the effects of the release and cleavage of CAP1 and other intracellular substrates after cytolysis may yield intriguing new insights in these pathologies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yexcr.2008.07.008.

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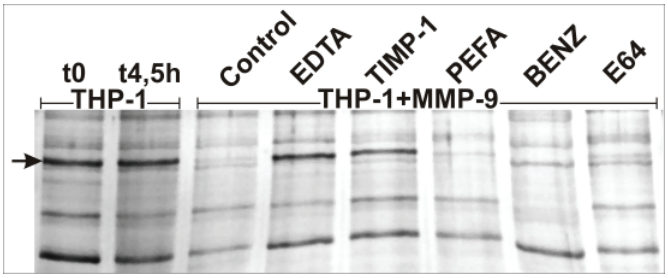
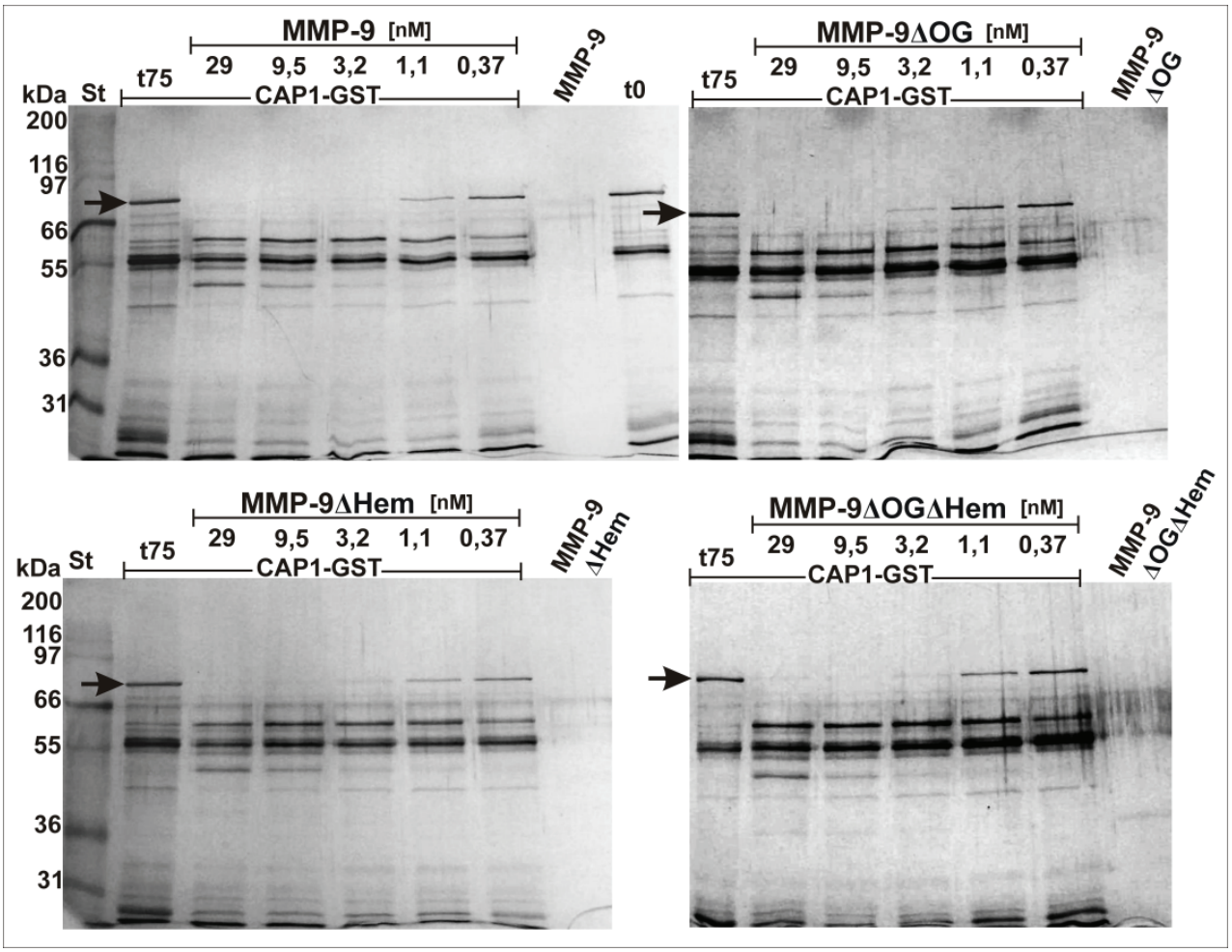
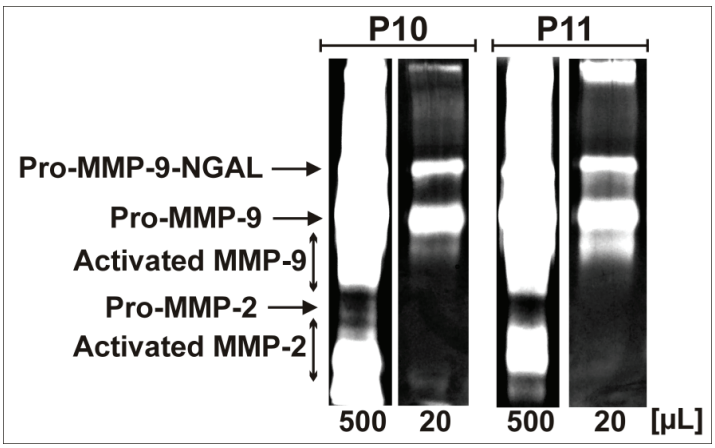
SUPPLEMENTARY MATERIAL



Supplementary Figure 1. Inhibition profile confirms cleavage of the 57 kDa protein by MMP-9. Culture medium of THP-1 cells containing the 57 kDa protein (arrow) was incubated for 4,5h in the absence or presence of 20 nM MMP-9, as well as various protease inhibitors. Addition of the general metalloproteinase inhibitor EDTA, or the MMP inhibitor TIMP-1 prevented degradation of the 57 kDa protein. In contrast, the serine protease inhibitors Pefabloc® (PEFA) and benzamidine (BENZ), as well as the cysteine protease inhibitor E64 did not inhibit the cleavage. Analysis of culture medium of THP-1 cells before incubation with MMP-9 is marked as t0. THP-1 culture medium after 4,5h of incubation without MMP-9 is indicated by t4.5h.



Supplementary Figure 2. Characterization of CAP1 cleavage with MMP-9 deletion mutants. Recombinant GST-tagged CAP1 (315 ng, 78 kDa) (arrows) was incubated during 75 min. in the absence or presence of various concentrations of activated MMP-9 variants: full-length MMP-9, MMP-9 with deletion of the O-glycosylated domain (MMP-9ΔOG), of the hemopexin domain (MMP-9ΔHem), and with deletion of both the hemopexin and O-glycosylated domains (MMP-9ΔOGHem). CAP1-GST was cleaved efficiently by MMP-9 and this cleavage is characterized by a CC_{50} of 0.5 nM. All MMP-9 variants cleaved CAP1 at equivalent concentrations. As a control for non-specific fragment bands, all MMP-9 variants were also incubated without the substrate. Recombinant CAP1 before and after 75 min. of incubation without MMP-9 variants is indicated by t0 and t75, respectively. The molecular mass standard is marked by St.



Supplementary Figure 3. Clarification of the gelatin zymograms of the urines of patients P10 and P11. Urine samples from patients P10 and P11 (500 µL) were prepurified on gelatin-Sepharose beads. To avoid overloading (*cf.* Figure 8, Panel D), 25 times less or the equivalent of 20 µl urine (instead of 500 µl) was analyzed by gelatin zymography and shown in parallel with the prepurifications of 500 µl. Pro-MMP-9-NGAL is neutrophil MMP-9 complexed with gelatinase B-associated lipocalin (NGAL).

CHAPTER 3. MULTIDIMENSIONAL DEGRADOMICS IDENTIFIES SYSTEMIC AUTOANTIGENS AND INTRACELLULAR MATRIX PROTEINS AS NOVEL GELATINASE B/MMP-9 SUBSTRATES

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Multidimensional degradomics identifies systemic autoantigens and intracellular matrix proteins as novel gelatinase B/MMP-9 substrates†

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The action radius of matrix metalloproteinases or MMPs is not restricted to massive extracellular matrix (ECM) degradation, it extends to the proteolysis of numerous secreted and membrane-bound proteins. Although many instances exist in which cells disintegrate, often in conjunction with induction of MMPs, the intracellular MMP substrate repertoire or degradome remains relatively unexplored. We started an unbiased exploration of the proteolytic modification of intracellular proteins by MMPs, using gelatinase B/MMP-9 as a model enzyme. To this end, multidimensional degradomics technology was developed by the integration of broadly available biotechniques. In this way, 100–200 MMP-9 candidate substrates were isolated, of which 69 were identified. Integration of these results with the known biological functions of the substrates revealed many novel MMP-9 substrates from the intracellular matrix (ICM), such as actin, tubulin, gelsolin, moesin, ezrin, Arp2/3 complex subunits, filamin B and stathmin. About 2/3 of the identified candidates were autoantigens described in multiple autoimmune conditions and in cancer (*e.g.* annexin I, nucleolin, citrate synthase, HMGB1, α-enolase, histidyl-tRNA synthetase, HSP27, HSC70, HSP90, snRNP D3). These findings led to the insight that MMPs and other proteases may have novel (immuno)regulatory properties by the clearance of toxic and immunogenic burdens of abundant ICM proteins released after extensive necrosis. In line with the extracellular processing of organ-specific autoantigens, proteolysis might also assist in the generation of immunodominant 'neo-epitopes' from systemic autoantigens. The study of proteolysis of ICM molecules, autoantigens, alarmins and other crucial intracellular molecules may result in the discovery of novel roles for proteolytic modification.

Introduction

Matrix metalloproteinases or MMPs constitute a family of Zn^{2+}-dependent proteases with a widespread substrate collection, ranging from extracellular matrix (ECM) proteins to signaling molecules, cytokines, chemokines, hormones as well as a kaleidoscope of membrane-associated substrates.[1,2] Although MMPs first came into the spotlights as ECM wreckers, recent evidence shows that they also degrade some intracellular matrix (ICM) proteins.[3–6] These findings suggest that intracellular substrates may be proteolyzed into "remnant epitopes", generating immunodominant epitopes for autoreactive T-cells, in this way initiating or exacerbating systemic autoimmunity.[7] Alternatively, degradation of abundant ICM proteins may be critical to avoid (actin) toxicity and immunogenicity after extensive cell death. Indeed, after cell necrosis, actin monomers may enter the systemic circulation and form long filaments together with coagulation factor Va, triggering disseminated intravascular coagulation if not rapidly cleared.

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Insight, innovation, integration

Proteolytic modification of intracellular substrates by extracellular proteases is an innovative concept in protease research. The aim of this study was to contribute to this new domain by characterizing the proteolysis of intracellular proteins by matrix metalloproteinases (MMPs) using unbiased, high-throughput approaches. By the integration of basic biochemical techniques, a multidimensional degradomics technology platform was applied to THP-1 cytosol with gelatinase B/MMP-9 as a model enzyme. About 70 intracellular proteins were identified as MMP-9 candidate substrates. Integration of these identifications with current biological knowledge about the substrates led to the insight that MMPs and other extracellular proteases may have novel (immuno)regulatory properties by the removal and modification of toxic and possibly immunogenic intracellular contents released into the extracellular space after extensive necrosis.

Functional organ decompensation may culminate into severe pathological conditions such as hepatic necrosis, adult respiratory distress syndrome (ARDS) and septic shock.[8–12] Finally, in acute necrotic conditions such as tumor lysis syndrome, the high tumor load and/or aggressive chemotherapy cause rapid tumor cell necrosis and a massive release of intracellular proteins into the systemic circulation.[13] As the resulting metabolic imbalances may lead to (fatal) multi-organ failure, rapid removal of the overload of toxic and immunogenic intracellular contents might be crucial for survival.

In this context, the aims of the present study were to identify novel intracellular MMP targets and to answer the question whether a previously undescribed task of MMPs may reside in the proteolytic modification of ICM proteins and (systemic) autoantigens. Extracellular proteolysis of extracellular matrix proteins, *e.g.* collagens,[14] or other secreted molecules, *e.g.* myelin basic protein[15] and insulin,[16] by the inflammatory protease gelatinase B/MMP-9 has previously been shown to contribute to organ-specific autoimmune diseases, respectively, rheumatoid arthritis, multiple sclerosis and diabetes. In many systemic autoimmune diseases, *e.g.* systemic lupus erythematosus (SLE), the autoantigens are ubiquitous intracellular proteins. Hence, we used MMP-9 as a model proteolytic enzyme to investigate whether the link between extracellular proteolysis and organ-specific autoimmune diseases may be extended to systemic autoimmunity.

In order to investigate the cleavage of intracellular proteins by MMPs, we tried to expand the limited list of intracellular MMP substrates by high-throughput degradomic identification of MMP-9 targets in the cytosolic fraction of THP-1 cells. Degradomics aims at characterizing the natural substrate repertoire or 'degradome' of a protease in a cell, tissue or organism.[17] In the present study, two multidimensional degradomic approaches were used as unbiased tools to explore the intracellular MMP-9 degradome. In the first dimension of the two-dimensional degradomics approach or '2D-degradomics', ion exchange chromatography (IEC) separated the THP-1 proteins by their net charge and/or isoelectric point (pI), whereas in the second dimension, potential substrates were separated by molecular weight upon SDS-PAGE analysis. In a second approach termed 'fragment degradomics', the THP-1 protein pool was first cleaved and low-molecular weight 'fragment fractions' were subsequently enriched by centrifugal filtration and concentration to visualize generated fragments or low-molecular weight substrates upon SDS-PAGE analysis. This straightforward, inexpensive and broadly applicable technology allowed us to isolate numerous candidate substrate (fragment) bands, ~70 of which were identified as potential MMP-9 substrates. As proofs-of-principle, various substrates were corroborated as previously identified gelatinase B (candidate) substrates by literature searches. Novel candidate substrates were confirmed by Western blot analysis of digested cytosol or by *in vitro* cleavage assays of the recombinant or purified substrate.

Remarkably, integration of the results generated by the multidimensional degradomics technology with the current state of biological knowledge in the literature revealed many novel MMP-9 substrates from the ICM. In addition, 2/3 of the identified candidates were autoantigens described in one or multiple autoimmune conditions and in cancer. These findings lead to the insight that MMPs and other proteases may have novel (immuno)regulatory properties by the removal of the toxic and immunogenic burden of abundant ICM proteins released after extensive necrosis and/or by the generation of immunodominant 'neo-epitopes' from systemic autoantigens.

Results

Development of two-dimensional degradomics or '2D-degradomics'

Recently, we identified a cytoskeletal protein, adenylyl cyclase-associated protein-1 or CAP1, as a highly efficient intracellular MMP-9 substrate by a one-dimensional degradomics approach on dying THP-1 cells.[6] Additional candidate substrates were discerned, but identification was not possible due to their low abundancy and/or overlap with other protein bands. In order to reduce the complexity of the initial protein pool and visualize potential MMP-9 substrates, an extra biochemical dimension was added to the previous one-dimensional approach. During 2D-degradomics, the proteins are first separated according to their net charge and/or pI by subsequent cation and anion exchange chromatography (CEC and AEC) (Fig. 1A). Before chromatography, the pH of the protein mixture is increased to pH 9.2 to generate a net negative charge in the majority of the proteins. Alkaline proteins are bound and eluted by the first CEC round. CEC flow through fractions are pooled and further fractionated by AEC chromatography. All AEC and CEC elution fractions, as well as the AEC flow through fractions, are dialyzed to remove the salt overload and concentrated. Next, each substrate fraction is incubated in the absence or presence of activated MMP-9. In the second dimension, digested and undigested fractions are separated by molecular weight upon SDS-PAGE analysis. Disappearing or decreasing of protein bands, or appearance of novel fragment bands after incubation with MMP-9 point to potential substrate proteins. The IEC fractions with the differential bands are then electroblotted onto PVDF for identification of the candidate substrate and/or cleavage site by Edman degradation. As many intracellular proteins are NH_2-terminally blocked, an alternative is to cut the differential bands out of the gels for identification by tandem mass spectrometry (MS/MS) after in-gel tryptic digest. Finally, a literature search is done to search for known MMP-9 substrates. Novel MMP-9 substrates are confirmed by cleavage of the recombinant or purified substrate *in vitro* or by Western blot analysis of digested and undigested cytosol with a substrate-specific antibody.

Application of 2D-degradomics to THP-1 cytosol unveils multiple MMP-9 candidate substrates

THP-1 cytosol was extracted by ultracentrifugation at pH 9.2 and 2D-degradomics was applied as described above and in the section of 'Materials and Methods'. About 80 IEC fractions were dialyzed, concentrated 2–10 times and incubated in the absence or presence of 100 nM MMP-9 for 24 h. The digested fractions were then analyzed on multiple SDS-PAGE gels, a representative gel of which is shown as an illustration in ESI, Fig. A.† Hence, with an estimated average of ~25 protein bands per fraction, a total number of

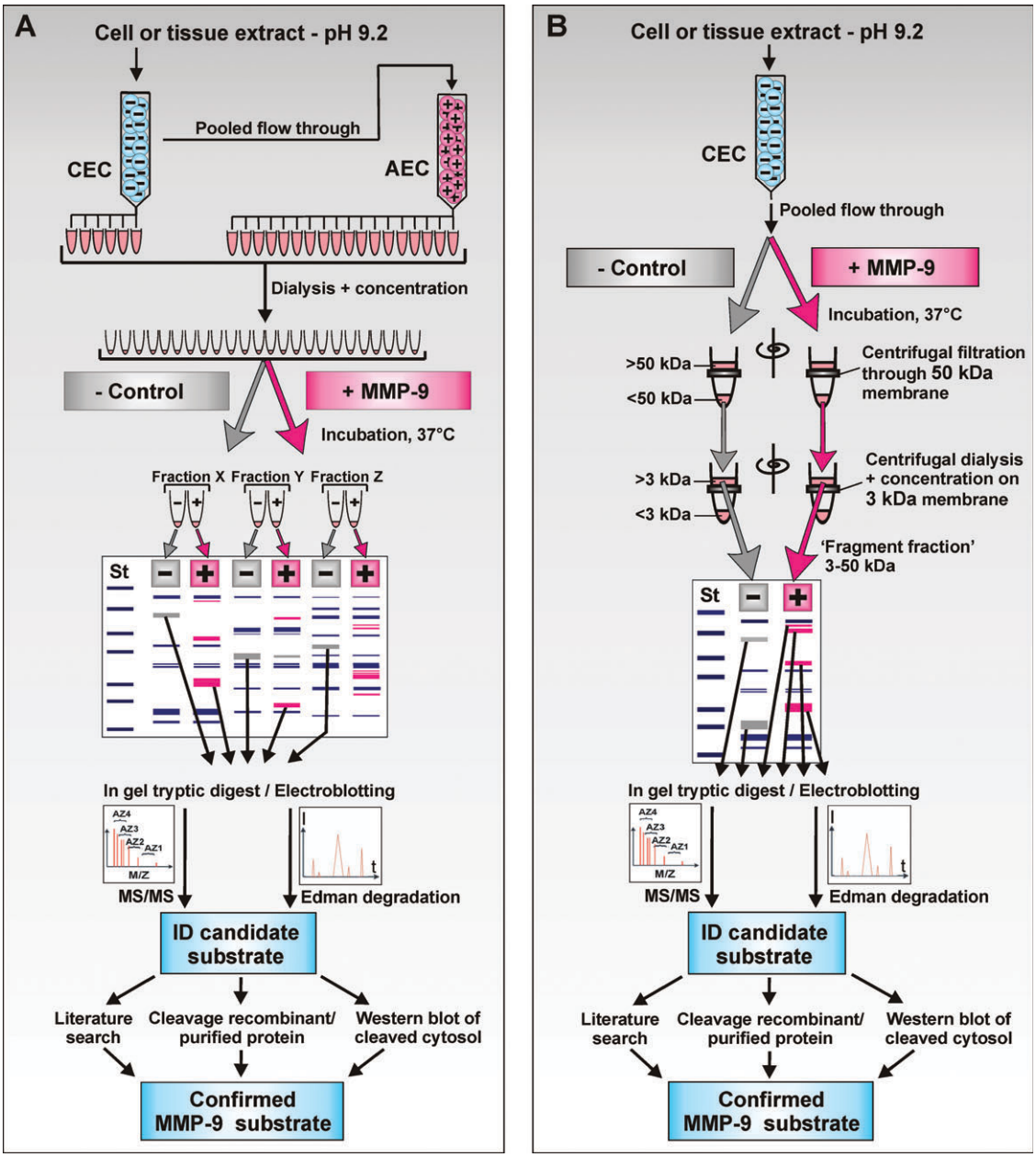

Fig. 1 *Flow charts of 2D-degradomics and fragment degradomics*. (A) Before *2D-degradomics*, the pH of the cell or tissue extract to be analyzed is first adjusted to pH 9.2, creating a net negative charge in the bulk of the proteins. In the first dimension, the protein mixture is applied to a cation exchange chromatography column (CEC) that binds and fractionates the alkaline proteins (with a positive charge at pH 9.2). The flow through fractions with the negatively charged proteins are pooled and fractionated by anion exchange chromatography (AEC). All resulting elution and flow through fractions are dialyzed to remove the salts, concentrated and incubated separately in the absence or presence of MMP-9. In the second dimension, cleaved and uncleaved fractions are further fractionated by molecular mass upon SDS-PAGE analysis. Protein bands that disappear or decrease after incubation with MMP-9 or novel fragments that appear indicate potential MMP-9 substrates. These differential bands are cut out of the gels and identified by MS/MS after in-gel tryptic digestion. Alternatively, the analyzed fractions are electroblotted onto PVDF and identified by Edman degradation. Finally, a literature search is performed to seek for substrates that have already been described. Other candidate substrates are confirmed biochemically by cleavage of the recombinant or purified substrate *in vitro* or by Western blot analysis of cleaved and uncleaved cytosol with substrate-specific primary antibodies. (B) Before *fragment degradomics*, the pH of the cell or tissue extract/fraction to be analyzed is brought to pH 9.2, and subjected to cation exchange chromatography (CEC), as described for 2D-degradomics. The pooled CEC flow through fractions are neutralized and incubated in the absence or presence of 100 nM MMP-9 for 24 h. Next, the 'fragment fractions' (3–50 kDa) are enriched on micro-spin columns by centrifugal filtration through a 50 kDa cut-off membrane, and subsequent centrifugal dialysis and concentration of the filtrate on a 3 kDa limiting membrane. Finally, the concentrated 'fragment fractions' are analyzed on SDS-PAGE to detect MMP-9-generated differential bands. Protein bands that disappear or decrease after incubation with MMP-9 or novel fragments that appear are potential MMP-9 substrates. These differential bands are cut out of the gels and identified by MS/MS after in-gel tryptic digests. Alternatively, the analyzed fractions are electroblotted onto PVDF and identified by Edman degradation. Finally, a literature search is performed to seek for known substrates. Other candidate substrates are confirmed biochemically by cleavage of recombinant or purified substrates *in vitro* or by Western blot analysis of cleaved and uncleaved cytosol with substrate-specific primary antibodies.

Table 1 Intracellular MMP-9 (candidate) substrates identified in THP-1 cytosol by '2D-degradomics' and 'fragment degradomics'

Protein symbol	Protein name	Swiss prot.[a]	# ID[b]	M_r^{exp}(S)[c]	M_r^{exp}(F)[c]	M_r^{th}(S)[c]	Score[d]	Seq. cov. (%)[e]	# Pept[f]	Confirmation[g]	Subcellular localization	(Putative) function
ACT	Actin	ND	3S + 1F	42	39	42	176/64/76/269	37/26/32/34	9(3)/8(1)/7(2)/12(2)	WB, IVC, CS	Cytoplasm, cytoskeleton, cell projection	Vital for cell morphogenesis and motility, endocytosis, phagocytosis and cytokinesis
ACTB	β-Actin	P60709	2S + 2F	42	40, 25	41	268/94/207/121	50/33/45/34	12(3)/10(2)/12(3)/6(2)			
ACTG	γ-Actin	P63261	2S + 4F	42	39, 29, 27, 12	42	110/67*/143/266/178/252	29/30/38/36/39/36	11(4)/7(1)/11(3)/9(3)/8(2)/16(2)			
ARP2	Actin-related protein 2	P61160	1S	45	—	45	131	30	12(1)	IVC	Cytoplasm, cytoskeleton, cell projection	ATP-binding component of the Arp2/3 complex which mediates the formation of branched F-actin networks
ARPC1A	Actin-related protein 2/3 complex subunit 1A	Q92747	—	—	—	42	—	—	—	IVC, CS		Actin-binding component of the Arp2/3 complex which mediates the formation of branched F-actin networks
ARPC2	Actin-related protein 2/3 complex subunit 2	O15144	1S	34	—	34	67*	32	14(1)	IVC		
ARPC3	Actin-related protein 2/3 complex subunit 3	O15145	—	—	—	21	—	—	—	IVC		
ARPC4	Actin-related protein 2/3 complex subunit 4	P59998	—	—	—	20	—	—	—	IVC		
ARPC5L	Actin-related protein 2/3 complex subunit 5-like protein	Q9BPX5	—	—	—	17	—	—	—	IVC, CS		
ARPC5	Actin-related protein 2/3 complex subunit 5	O15511	—	—	—	16	—	—	—	IVC		
ALDOA	Aldolase A	P04075	2S + 1F	42	30	39	174/234/187	23/30/28	7(3)/11(4)/11(2)	—	Cytoplasm	Glycolytic enzyme that catalyzes the reversible conversion of fructose-1,6-bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate
ALIX	Apoptosis-linked-gene-2-interacting-protein X	Q8WUM4	2S	85	—	97	179/209	27/24	20(3)/18(4)	—	Cytoplasm (melanosome)	Control of the production of and trafficking through endosomes called multivesicular bodies; regulation of caspase-dependent and caspase-independent cell death
ANXA1	Annexin I	P04083	1F	—	33	40	221	38	15(3)	WB	Nucleus, cytoplasm, cell projection, cell surface	Calcium/phospholipid-binding protein which promotes membrane fusion and is involved in exocytosis; regulation of phospholipase A2 activity; inhibition of neutrophil extravasation and induction of neutrophil apoptosis
ARCH	Archease	Q8IWT0	1F	—	27	18	79*	18	3(1)	—	—	Putative chaperone or modulator of proteins involved in DNA/RNA processing
ACLY	ATP citrate lyase	P53396	2S	115	—	120	427/112	33/23	35(4)/22(2)	—	Cytoplasm	Synthesis of cytosolic acetyl-CoA; central role in *de novo* lipid synthesis; role in biosynthesis of acetylcholine in nervous tissue
BID	BH3 interacting domain death agonist	P55957	1F	—	24	27	166	38	7(2)	—	Cytoplasm, mitochondrial membrane	Major proteolytic product p15 BID: release of cytochrome c; induction of ICE-like proteases and apoptosis; countering the protective effect of Bcl-2
CAP1	Adenylyl cyclase-associated protein-1	Q01518	3F	—	27, 25, 18	52	281/228/70	41/13/16	8(3)/5(2)/9(3)	6	Cytoplasm, cell membrane	Enhancement of actin filament turnover; roles in cell morphology, migration and endocytosis

Table 1 (*continued*)

Protein symbol	Protein name	Swiss prot.[a]	# ID[b]	M_r^{exp}(S)[c]	M_r^{exp}(F)[c]	M_r^{th}(S)[c]	Score[d]	Seq. cov. (%)[e]	# Pept[f]	Confirmation[g]	Subcellular localization	(Putative) function
CAP-G	Actin regulatory protein CAP-G	P24452	S + F	40	30	35	103/223	14/18	5(3)/7(2)	—	Cytoplasm, nucleus, melanosome	Reversible blocking of actin filament barbed ends without filament severing; may have important roles in macrophage function and regulation of cytoplasmic and/or nuclear structures through interactions with actin; potential DNA binding
CA-II	Carbonic anhydrase II	P00918	1S	30	—	29	162	20	5(2)	—[h]	Cytoplasm	Reversible hydration of carbon dioxide; essential for bone resorption and osteoclast differentiation
CS	Citrate synthase	O75390	1S	46	—	52	150	21	10(3)	IVC	Mitochondria	Catalytic activity: Acetyl-CoA + H_2O + oxaloacetate = citrate + CoA
CypA	Cyclophilin A	P62937	1S	18	—	18	131	51	15(2)	18	Cytoplasm	Acceleration of protein folding, catalysis of *cis–trans* isomerization of peptidyl–prolyl bonds in oligopeptides; pro-inflammatory functions after secretion
CypE	Cyclophilin E	Q9UNP9	1F	—	18	33	64*	22	3(1)	—	Nucleus	Acceleration of protein folding, catalysis of *cis–trans* isomerization of peptidyl–prolyl bonds in oligopeptides; binding of RNA with potential role in pre-mRNA splicing
CysRS	Cysteinyl-tRNA synthetase	P49589	1F	72	—	83	181	22	18(3)	—	Cytoplasm	Catalytic activity: ATP + L-cysteine + CysRS = AMP + diphosphate + L-cysteinyl-CysRS
eEF1-α	Elongation factor 1-α1	P68104	2F	—	18, 11	50	72/82	26/37	11(1)/6(1)	—	Cytoplasm	Promotion of the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis
eEF-2	Elongation factor 2	P13639	2S	95	—	95	197/166	25/10	24(3)/7(2)	—	Cytoplasm	Promotion of the GTP-dependent translocation of the nascent protein chain from the A-site to the P-site of the ribosome
ENO1	α-Enolase	P06733	2S	47	—	47	99/123	27/22	10(3)/10(4)	—	Cytoplasm, cell membrane or myofibril, sarcomere, M-band	Glycolytic enzyme; role in growth control, hypoxia tolerance and allergic responses; receptor and activator of plasminogen
ERp29	Endoplasmic reticulum protein ERp29	P30040	1S	30	—	29	83	14	3(1)	—	ER lumen	Important in the processing of secretory proteins within the ER
eIF-5A	Eukaryotic translation initiation factor 5A	P63241	1S	19	—	17	155	59	7(2)	—	Cytoplasm	Promotion of the formation of the first peptide bond during protein biosynthesis
Ezrin	Ezrin	P15311	4S	75/55	—	69	143/445/100/279	12/30/16/30	8(2)/21(5)/7(2)/21(4)	—	Cytoplasm, cell membrane, cell projections	Cross-linking of cortical actin filaments and plasma membranes; roles in ECM interactions, cell–cell communication, apoptosis, carcinogenesis and metastasis
Fascin	Fascin	Q16658	1S	54	—	54	223	33	18(3)	—	Cytoplasm, cytoskeleton, cell projection	Organization of F-actin into bundles with a minimum of 4.1 : 1 actin : fascin ratio
FTL	Ferritin light chain	P02792	1S	24	—	20	106	45	8(2)	—	Cytoplasm	Storage of iron in a soluble, non-toxic, readily available form

Table 1 (*continued*)

Protein symbol	Protein name	Swiss prot.[a]	# ID[b]	M_r^{exp}(S)[c]	M_r^{exp}(F)[c]	M_r^{th}(S)[c]	Score[d]	Seq. cov. (%)[e]	# Pept[f]	Confirmation[g]	Subcellular localization	(Putative) function
FLN-B	Filamin B	O75369	1S	227	—	227	71	8	19(2)	—	Cytoplasm, cytoskeleton	Crosslinking of actin filaments to form orthogonal networks; connection of membrane proteins to the actin cytoskeleton
GDH/6PGL	GDH/6PGL endoplasmic bifunctional protein	O95479	1S	89	—	89	419	40	32(6)	—	ER lumen	Oxidizes glucose-6-phosphate and glucose, as well as other hexose-6-phosphates.
GDI2	GDP dissociation inhibitor 2	Q5SX88	1S	47	—	46	96	30	10(1)	—	Cytoplasm	Rab GDP-dissociation inhibitor activity
β-G1	β-Glucuronidase	P08236	1S	85	—	75	93	11	9(3)	—	Lysosome	Catalytic activity: β-D-glucuronoside + H_2O = an alcohol + D-glucuronate; important role in degradation of dermatan and keratan sulfates
GSN/ADF	Gelsolin	P06396	1S	81	—	81	385	34	18(3)	3, 4	Cytoplasm, cytoskeleton	F-actin capping and severing; nucleation of F-actin assembly
GSTP1	Glutathione S-transferase P	P09211	1S	25	—	19	89	28	6(1)	—	Cytoplasm	Conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles
HDGF	Hepatoma-derived growth factor	P51858	S + F	45	36	27	223/89	44/20	11(3)/7(2)	—	Cytoplasm	Heparin-binding protein with mitogenic activity for fibroblasts
HMGB1	High-mobility group protein B1	P09429	S + F	28	8	25	152/205	34/28	8(3)/6(2)	WB	Nucleus	Intracellular function: preferential binding of ss-DNA and unwinding of ds-DNA, regulating transcription; extracellular function: pro-inflammatory cytokine, alarmin
HMGB2	High-mobility group protein B2	P26583	1S	24	—	22	78	43	9(2)	—	Nucleus	Intracellular function: preferential binding of ss-DNA and unwinding of ds-DNA, regulating transcription
Jo-1/HisRS	Histidyl-tRNA synthetase	P12081	1S	57	—	57	189	33	18(3)	IVC, CS	Cytoplasm	Catalytic activity: ATP + L-histidine + HisRS = AMP + diphosphate + L-histidyl-HisRS
HSP27	Heat shock protein 27	P04792	S + F	30	12	22	98/63	25/16	6(2)/2(1)	—	Cytoplasm, cytoskeleton, nucleus	Molecular chaperone involved in stress resistance and actin organization (actin capping)
HSC70	Heat shock cognate protein 70	P11142	1S	70	—	71	182	30	14(3)	—	Cytoplasm, cytoskeleton, nucleus	Stabilization of pre-existing proteins against aggregation; mediation of the folding of newly translated polypeptides in the cytosol and within organelles
HSP90α	Heat shock protein 90α	P07900	S + F	85	66	85	238/409	22/28	16(3)/12(4)	18, 19	Cytoplasm, cytoskeleton, nucleus, ER lumen, mitochondria cell surface	Molecular chaperone with ATPase activity; F-actin bundling and cross-linking
HSP90β	Heat shock protein 90β	P08328	1S	85	—	83	191	33	23(4)	—		
IQGAP1	IQ motif containing GTPase activating protein 1	A4QPB0	2S + 1F	190, 120	85	189	193/374/362	19/23/29	29(4)/38(5)/49(5)	—	Cell leading edge, cytoplasm	Actin crosslinking/bundling; E-cadherin-mediated cell–cell contacts; microtubule capture/polarity; cell motility and invasion; phagocytosis

Table 1 (*continued*)

Protein symbol	Protein name	Swiss prot.[a]	# ID[b]	M_r^{exp}(S)[c]	M_r^{exp}(F)[c]	M_r^{th}(S)[c]	Score[d]	Seq. cov. (%)[e]	# Pept[f]	Confirmation[g]	Subcellular localization	(Putative) function
ISG15	ISG15 ubiquitin-like modifier	Q5SVA4	2S	16	—	18	97/65*	23/23	4(2)/4(2)	—	Secreted/ released, cytoplasm, cytoskeleton	Conjugation of proteins with potential roles in antiviral response; regulation of cell growth and carcinogenesis; cytokine function in stimulation of NK cell proliferation and increasing leukocyte toxicity in the presence of T cells
LACTB2	β-Lactamase-like protein 2	Q53H82	1F	—	33	33	105	36	13(1)	—	ND	Putative hydrolase activity and iron binding
LRP130	Leucine-rich PPR motif-containing protein	P42704	1S	160	—	158	119	15	25(4)	—	Nucleus, ER, mitochondrion	ssDNA/RNA binding protein with potential roles in RNA metabolism, gene transcription and gluconeogenesis
MAPRE1	Microtubule-associated protein RP	Q15691	1F	—	16	19	169	33	5(1)	—	Cytoplasm, microtubule network, centrosome	Microtubule formation and stabilization, promoting cell migration; binding and inhibition of the F-actin bundling and microtubule-associated protein APC
MSN	Moesin	P26038	3S + 5F	72, 62	62, 42, 38, 20	68	152/205/343/ 177/156/125/ 83/315	24/26/43/28/ 33/30/20/32	12(3)/17(2)/ 18(3)/13(3)/ 14(3)/21(2)/ 18(2)/21(3)	—	Cytoplasm, cell membrane, cell projections	Cross-linking of cortical actin filaments and plasma membranes; roles in ECM interactions, cell–cell communication, apoptosis, carcinogenesis and metastasis
NIT2	Nitrilase homolog 2	Q9NQR4	1F	—	33	31	90	24	7(2)	—	Cytoplasm	Cell growth inhibition, potential tumor suppressor
NCL	Nucleolin	P19338	1S	60	—	77	106	18	13(1)	WB	Nucleus, cell surface, (cytoplasm)	Regulation of RNA polymerase I transcription; folding and maturation of pre-ribosomal RNA; ribosome assembly; nucleo-cytoplasmic transport; histone chaperone activity; interaction with viruses at the cell membrane
NME1-NME2	Nucleoside diphosphate kinase	Q1WM23	1S	19	—	30	106	25	6(3)	—	Cytoplasm, cytoskeleton, nucleus	Catalytic activity: ATP + nucleoside diphosphate = ADP + nucleoside triphosphate
hNRP/ NAP1L1	Nucleosome assembly protein 1-like 1	P55209	S + F	50	48	45	209/173	17/15	8(3)/6(3)	—	Nucleus (melanosome)	Putative roles in chromatin formation and DNA replication, regulating cell proliferation
Orp150	Oxygen-regulated protein 150	Q9Y421	1S	150	—	150	229	23	23(6)	—	ER lumen, mitochondria	Molecular chaperone, cytoprotective after oxygen deprivation
PC4	Positive cofactor 4	P53999	1S	16	—	14	158	66	10(3)	—	Nucleus	Transcriptional coactivator, binding of ssDNA
PRDX2	Peroxiredoxin 2	P32119	1F	—	24	22	193	39	6(2)	—	Cytoplasm, cell membrane	Antioxidant by peroxidase activity, control of cytokine-induced peroxide levels which mediate signal transduction
PRDX6	Peroxiredoxin 6	P30041	1S	28	—	25	61*	31	7(1)	—	Cytoplasm, lysosomes	Antioxidant with peroxidase and phospholipase A2 activities
PGK1	Phosphoglycerate kinase 1	P00558	2S	45	—	45	157/293	21/47	6(2)/14(4)	—	Cytoplasm	Glycolytic enzyme; putative polymerase α cofactor protein
PURH	Bifunctional purine biosynthesis protein	P31939	1S	65	—	65	177	26	12(3)	—	Cytoplasm	Catalysis of the last two steps in the pathway for *de novo* synthesis of inosine 5′-monophosphate

Table 1 (*continued*)

Protein symbol	Protein name	Swiss prot.[a]	# ID[b]	M_r^{exp}(S)[c]	M_r^{exp}(F)[c]	M_r^{th}(S)[c]	Score[d]	Seq. cov. (%)[e]	# Pept[f]	Confirmation[g]	Subcellular localization	(Putative) function
RCL	c-Myc-responsive protein Rcl	O43598	1S	24	—	19	207	37	6(3)	—	Nucleus	Role during cellular proliferation and c-myc-mediated transformation; anti-apoptotic protein
Rho GDI α	Rho GDP dissociation inhibitor (GDI) α	P52565	2F	—	24	22	212/168	38/17	9(3)/5(2)	—	Cytoplasm	Regulates the GDP/GTP exchange reaction of the Rho proteins by
Rho GDI β	Rho GDP dissociation inhibitor (GDI) β	P52566	1S	30	—	23	57*	23	3(1)	—	Cytoplasm	inhibiting the dissociation of GDP, and the subsequent binding of GTP
snRNP D3	Small nuclear ribonucleoprotein Sm D3	P62318	1F	—	11	14	150	45	5(2)	—	Nucleus	Component of the spliceosome, involved in the nuclear processing of pre-mRNA
STMN1	Stathmin	P16949	2S + F	17	14	17	134/180/83	22/31/36	5(2)/5(2)/6(1)	ES, CS, WB	Cytoplasm, cytoskeleton	Regulation of microtubule dynamics by microtubule depolymerization and inhibition of polymerization
SAE2	SUMO-activating enzyme subunit 2	Q9UBT2	1S	95	—	71	124	16	9(2)	—	Nucleus	ATP-dependent activation of SUMO proteins and formation of thioester with a conserved cysteine residue on SAE2
TBCA	Tubulin-specific chaperone A	O75347	1S	16	—	13	103	19	3(2)	—	Cytoplasm, cytoskeleton	Tubulin-folding protein; involved in the early step of the tubulin folding pathway.
ThrRS	Threonyl-tRNA synthetase	P26639	1S	75	—	83	85	21	16(1)	—	Cytoplasm	Catalytic activity: ATP + L-threonine + tRNA(ThrRS) = AMP + diphosphate + L-threonyl-tRNA(ThrRS)
TUBA	α-tubulin	P68363	1S	50	—	50	208	25	13(3)	IVC, CS	Cytoplasm,	Heterodimers of α and β chains are
TUBB	β-tubulin	P07437	1S	50	—	50	168	28	18(2)		cytoskeleton, cell projection	the major constituents of microtubules
UBE2NL	Ubiquitin-conjugating enzyme E2N-like	Q5JXB2	1S	17	—	17	91	30	6(2)	—	Nucleus	ND, belongs to the ubiquitin-conjugating enzyme family
UBE2V1	Ubiquitin-conjugating enzyme E2 variant 1	Q13404	2S	17	—	16	113/92	40/38	7(3)/7(1)	—	Nucleus	Catalysis of non-canonical poly-ubiquitin chain synthesis; role in the activation of Nf-κB; transcriptional activation of target genes; role in the control of cell cycle and differentiation; role in error-free DNA repair pathway; contribution to cell survival after DNA damage
UMP-CMPk	UMP-CMP kinase	P30085	1S	25	—	22	75*	22	6(2)	—	Nucleus, cytoplasm	Catalysis of phosphoryl transfer from ATP to UMP and CMP

APC, adenomatous polyposis coli; AMP, adenosine monophosphate; ATP, adenosine triphosphate; CMP, cytidine monophosphate; **F-actin**, filamentous actin; **ds-DNA**, double-stranded DNA; ER, endoplasmic reticulum; GDH/6PGL, glucose 1-dehydrogenase/6-phosphogluconolactonase; ND, not defined; PPR, pentatricopeptide repeat cassette, ss-DNA, single-stranded DNA; UMP, uridine monophosphate. [a] SwissProt accession number (www.expasy.org). [b] Number of identifications as an MMP-9 substrate (S) or substrate fragment (F) by 2D-degradomics and fragment degradomics. [c] M_r^{exp}(S), experimentally determined average molecular mass of the candidate substrate; M_r^{exp}(F), experimentally determined average molecular mass of the substrate fragment(s); M_r^{th}, theoretical average molecular mass of the candidate substrate. [d] Score as determined by the Mascot software (www.matrixscience.com), * indicates that the score is non-significant (95% confidence level). [e] Sequence coverage (Seq. cov.) (%) as determined by MALDI-TOF/TOF. [f] Numbers of peptides identified by MALDI-TOF. The numbers of peptides that have been analysed by MALDI-TOF/TOF sequencing are shown between brackets. [g] Confirmation of an MMP-9 candidate substrate by Western blot analysis of digested cytosol with a substrate-specific antibody (Western blot, WB); cleavage of the recombinant/purified substrate *in vitro* (*in vitro* cleavage, IVC), additional identification by Edman sequencing (ES) and/or determination of cleavage sites (cleavage sites, CS). If a number is provided in this column, it refers to confirmation in the corresponding literature reference. [h] Not cleaved *in vitro*.

~2000 proteins were separated and visualized by 2D-degradomics. In this way, 100–200 potential MMP-9 substrates were detected. Subsequently, ~60 substrates were identified by MS/MS after in-gel tryptic digests, whereas one substrate fragment was also identified by Edman degradation (Table 1).

Additional potential MMP-9 substrates identified by 'fragment degradomics'

In an alternative degradomic approach refered to as 'fragment degradomics' (Fig. 1B), the total protein mixtures (pooled CEC flow through fractions) were incubated in the absence or presence of 100 nM MMP-9 for 24 h. Next, the 'fragment fractions' (3–50 kDa) were enriched on micro-spin columns by subsequent centrifugation through a 50 kDa cut-off membrane, followed by centrifugal dialysis and concentration of the filtrate on a 3 kDa membrane. Finally, the concentrated retentate was analyzed on SDS-PAGE to detect MMP-9-generated differential bands as exemplified in ESI, Fig. B.† The fragments of potential MMP-9 substrates were cut out of the gel and identified by MS/MS. Substrates (*e.g.* stathmin and adenylyl cyclase-associated protein-1) that were previously identified by 2D-degradomics were also detected by fragment degradomics (Table 2). In addition, seven novel candidate substrates were identified by this fragment-enriching approach.

Confirmation of the cleavage of intracellular candidate substrates by MMP-9

As a 'proof-of-principle' of both multidimensional degradomic approaches, our data were confronted with an in-depth literature search for known MMP-9 substrates. Indeed, the intracellular MMP-9 substrate CAP1 that was discovered by our one-dimensional degradomic approach[6] was identified by both multidimensional methods. Of the 69 candidate substrates discovered by 2D-degradomics and fragment degradomics, 14 proteins (20%) were previously identified as MMP-9 (candidate) substrates (Table 1 and 3) by highly diverging approaches, ranging from 2-dimensional gel electrophoresis to a label-free ultra performance liquid chromatography (UPLC)-based approach.[3,4,20,21] Hence, this 20% overlap with other methods is a first corroboration of our approaches. In addition, about half of the substrates listed in Table 1 are known MMP (candidate) substrates (Table 3). Confirmed MMP substrates (discovered by proteomic or other approaches and validated biochemically or by *in vivo* cleavage) are considered to be high-confidence MMP-9 candidate substrates. Potential substrates that were identified both as a substrate (band disappearing or decreasing after incubation with MMP-9) and as a fragment (a novel protein band appearing in the presence of MMP-9), or that were identified in many fractions, also represent strong candidate substrates. However, we acknowledge that these high-confidence candidate substrates need to be confirmed *in vivo* or by biochemical assays with purified MMP-9 before being considered as novel MMP-9 substrates. Selection of candidate substrates for biochemical validation was based on the propensity of the protein to be an interesting physiological target. The availability of recombinant or purified proteins and primary antibodies of good quality was another crucial factor. In addition, both strong and low confidence candidate substrates (single identification) were chosen for validation.

THP-1 cytosol was incubated in the absence or presence of various concentrations of activated MMP-9, supplemented or

Table 2 MMP-9 (candidate) substrates identified in THP-1 cytosol by 'fragment degradomics'

Candidate substrates previously identified by 2D-degradomics
Adenlyl cyclase-associated protein-1
Stathmin
Novel candidate substrates identified by fragment degradomics
Carbonic anhydrase II
Cyclophilin A
Glutathion S-transferase P
ISG15 ubiquitin-like modifier
Peroxiredoxin 6
Tubulin-specific chaperone A
UMP-CMP kinase

Table 3 Known MMP-9 or MMP (candidate) substrates identified by 2D-degradomics and fragment degradomics

(Candidate) substrate	MMP(s) responsible[a]	Ref(s).
β-Actin	*MMP-9*	21
γ-Actin	*MMP-9*	21
Actin regulatory protein CAP-G	*MMP-9*, *MMP-2*, *-14*	18, 19, 21
Actin-related protein 2	*MMP-14*	18
Adenylyl cyclase-associated protein-1	**MMP-9**, **MMP-2**, **-8**, **-13**, *-14*	6, 18
Aldolase A	*MMP-9*, *MMP-14*	18, 21
Annexin I	*MMP-2*	19
Cyclophilin A	*MMP-9*, **MMP-14**	18, 21
Elongation factor 1-α1	*MMP-9*, *MMP-14*	18, 21
Elongation factor 2	*MMP-14*	18
Endoplasmic reticulum protein Erp29	*MMP-14*	18
α-Enolase	*MMP-2*	19
Eukaryotic translation Initiation factor 5A	*MMP-14*	18
Ezrin	*MMP-14*	18
Gelsolin	**MMP-9**, **MMP-1**, **-2**, **-3**, **-14**	3, 4
High-mobility group protein B1	*MMP-14*	18
High-mobility group protein B2	*MMP-2*, *-14*	18, 19
Heat shock cognate protein 70	*MMP-9*, *MMP-2*, *-14*	18, 19, 21
Heat shock protein 90α	**MMP-2**, **-14**	18, 19, 21
Heat shock protein 90β	*MMP-9*	21
Jo-1	*MMP-2*, *-14*	18, 21
Moesin	*MMP-9*, *MMP-14*	18, 21
Nucleolin	*MMP-2*	19
Nucleoside diphosphate kinase	*MMP-9*	21
Oxygen-regulated protein 150	*MMP-14*	18
Peroxiredoxin-6	*MMP-9*, *MMP-2*	20
Phosphoglycerate kinase 1	*MMP-9*, *MMP-14*	18, 21
Small nuclear ribonucleoprotein Sm D3	*MMP-14*	18
Threonyl-tRNA synthetase	*MMP-9*, *MMP-14*	18, 21
α-Tubulin	*MMP-9*, *MMP-14*	18, 21
β-Tubulin	*MMP-9*, *MMP-14*	18, 21

[a] MMP substrates that were only identified by a proteomic approach and not yet confirmed by biochemical assays or *in vivo* cleavage are considered MMP candidate substrates. MMP(s) cleaving biochemically *confirmed* substrates are shown in bold, whereas MMP(s) cleaving *candidate* substrates are shown in italics.

not with the metalloprotease inhibitors 1,10-*o*-phenanthroline or EDTA. Western blot analysis of these digestions with substrate-specific primary antibodies led to the confirmation of *annexin I*, *nucleolin*, *stathmin*, *HMGB1* and *β- and γ-actin* as novel intracellular MMP-9 substrates (Fig. 2). Interestingly, no HMGB1 cleavage was observed after Western blot analysis of unfractionated cytosol (data not shown). However, *in vitro* cleavage of HMGB1 by MMP-9 was confirmed by Western blot analysis of the concentrated IEC fraction in which it was identified during 2D-degradomics (Fig. 2). This underscores that multidimensional degradomics allows for the identification of lower abundancy and lower-affinity, but still physiologically interesting substrates, that may be missed with other methods that are restricted in their loading capacity.

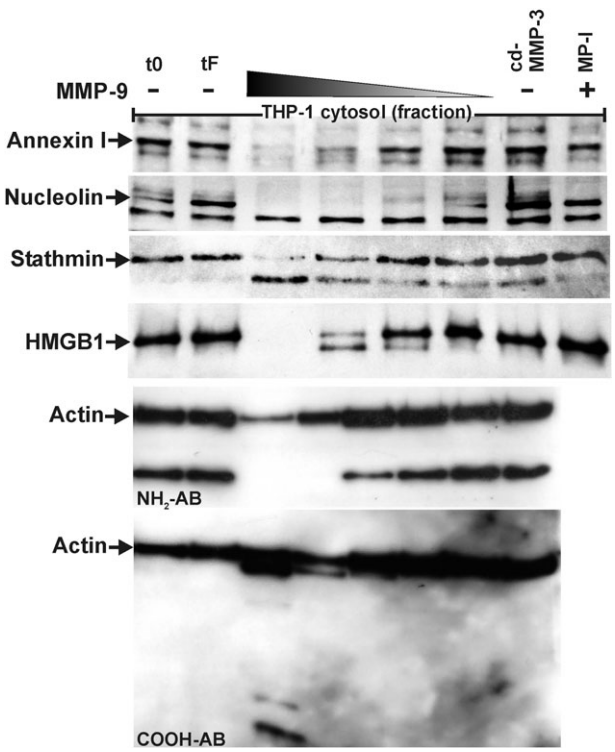

Fig. 2 *Confirmation of novel intracellular MMP-9 substrates by Western blot analysis.* THP-1 cytosol was incubated in the absence (tF, −) or presence (+) of activated MMP-9 (200–1.6 nM, decreasing from lane 3 to 6; 500–0.8 nM, decreasing from lane 3 to 7 for actin). Control incubations included incubation with 40 nM activated MMP-9 supplemented with 10 mM of the metalloprotease inhibitors 1,10-*o*-phenantroline or EDTA (MP-I), and addition of equivalent amounts of the catalytical domain of MMP-3 (cd-MMP-3, 2 nM), used to activate MMP-9. Western blot analysis with substrate-specific primary antibodies confirmed cleavage of the substrates annexin I, nucleolin, stathmin, HMGB1 and β- and γ-actin by MMP-9. To detect actin cleavage, antibodies against the NH_2-terminus (NH_2-AB) or against the COOH-terminus (COOH-AB) were used separately to discern NH_2- and COOH-terminal truncations. Analysis of the substrate before incubation is marked as t0. Incubation intervals were 4.5 h for annexin I, nucleolin and stathmin analysis, 18 h for HMGB1 and 24 h for actin cleavage.

For the cleavage assays the recombinant or purified candidate proteins were incubated with various MMP-9 concentrations, supplemented or not with the metalloprotease inhibitor EDTA and analysed by SDS-PAGE. In this way, we validated *α- and β-tubulin, α-, β- and γ-actin, citrate synthase*, the autoantigen *Jo-1* and most subunits of the *actin-related protein-2/3 (Arp2/3) complex* as intracellular MMP-9 targets (Fig. 3 and 4). Both rabbit skeletal muscle (α-actin, Fig. 3) and human non-muscle platelet actin (β- and γ-actin, Fig. 4) were cleaved by MMP-9. Human skeletal muscle actin (α-actin) and human non-muscle actin (β- and γ-actin) were cleaved with comparable efficiencies (data not shown), which is not surprising since the amino acid-homology between the three main actin isotypes is more than 90%. All subunits of the Arp2/3 complex were cleaved after long incubation intervals and with high enzyme/substrate ratios, except for actin-related protein 3 (ARP3) (Fig. 3 and 4). The subunits ArpC1A and ArpC5L, were cleaved most efficiently. Of the candidate substrates selected for validation by *in vitro* cleavage assays, only carbonic anhydrase II was not confirmed (data not shown), suggesting the action of a proteolytic cascade including MMP-9 in the IEC fraction.

When clear and abundant fragment bands were detected, the digested and undigested substrates were analyzed by SDS-PAGE and transferred to PVDF by electroblotting. Cleavage sites were determined by Edman sequencing of the fragment NH_2-termini (Fig. 4). In addition, cleavage sites may also be determined directly in electroblotted IEC fractions, as was done for stathmin (*cf*. Fig. 4E). Several protein bands were resistant to Edman sequencing (indicated by asterisks in Fig. 4), but others were identified by direct protein sequencing. When non-human purified proteins were used for *in vitro* cleavages and cleavage site determinations, all detected cleavage sites were in stretches of 100% amino acid identity between the human and other species. Likewise, the major actin cleavage site (Glu46-Met47) was determined in human platelet actin (a mixture of β- and γ-actin) in a region of 100% identity between the three main actin isotypes (α, β and γ).

Most MMP substrates are cleaved by more than one MMP due to the broad homology and redundancy in the MMP family.[2,18] Table 3 shows that proteolysis of intracellular substrates is not restricted to MMP-9. Hence, Western blot analysis and *in vitro* cleavage assays of several substrates were repeated with equal amounts of activated MMP-9, gelatinase A/MMP-2, the collagenases MMP-1, MMP-8 and MMP-13 and the catalytical domain of stromelysin-1/cd-MMP-3 (Fig. 5). The tested substrates were all cleaved by various MMPs, with a variable substrate-dependent efficiency pattern among the MMPs used. Carbonic anhydrase II, which was not clearly proteolyzed by MMP-9 *in vitro*, was cleaved by MMP-13, confirming it as an MMP target. Interestingly, MMP-13 was able to cleave all the tested intracellular proteins, most of these with high efficiency (Fig. 5).

Degradation of the intracellular matrix (ICM) by MMPs

As recent studies showed that MMPs also degrade various proteins of the intracellular matrix (ICM), [3–6] we examined whether the candidate substrates identified by multi-dimensional degradomics (Table 1) include ICM proteins. A literature search was performed to investigate which molecules are associated with the cytoskeleton, more specifically

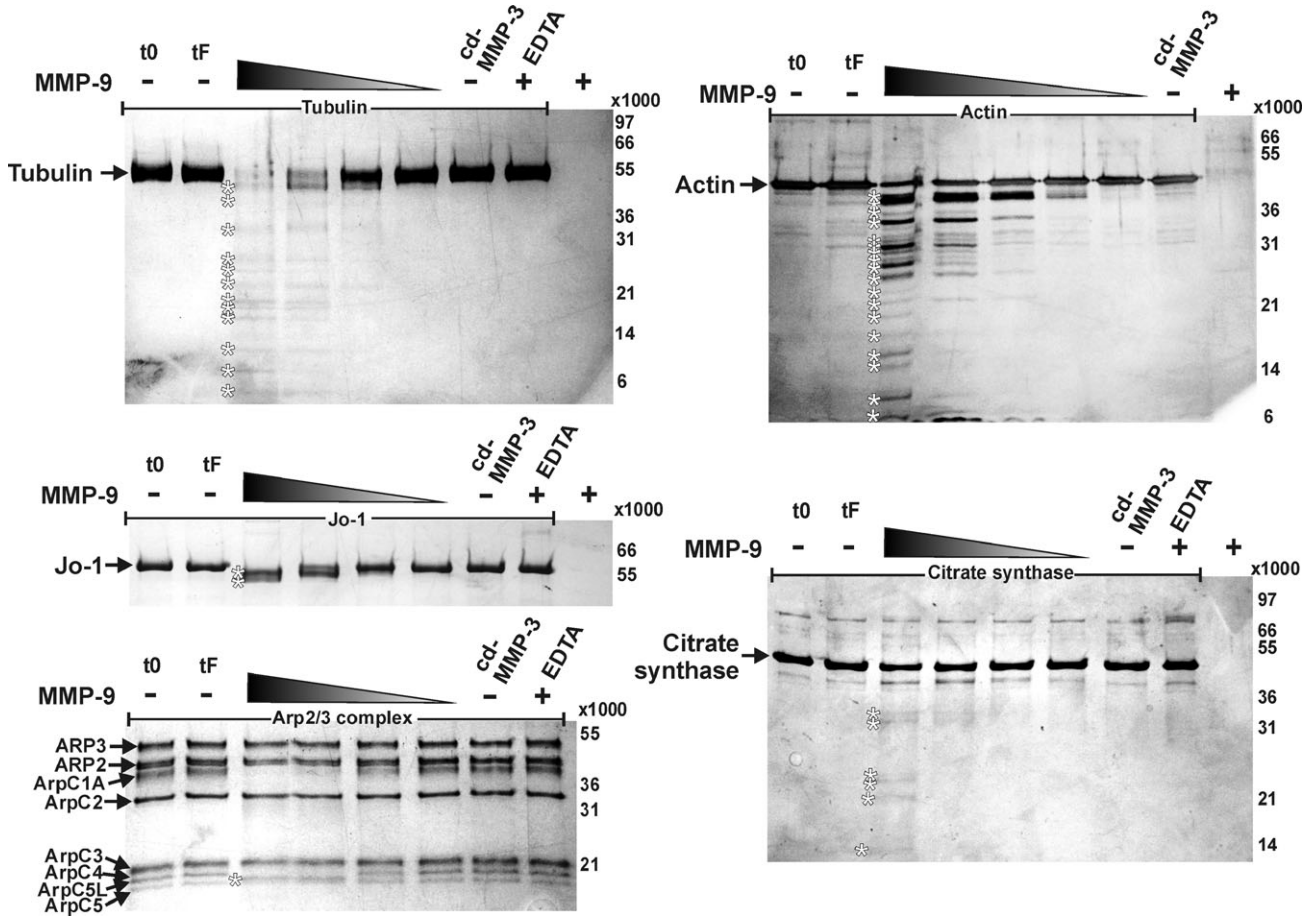

Fig. 3 *Confirmation of novel intracellular MMP-9 substrates by in vitro cleavage of recombinant or purified proteins.* Proteins were incubated in the absence (tF, −) or presence (+) of various concentrations of activated MMP-9 (decreasing from lane 3 to 6 or 7). Control incubations included incubation with activated MMP-9 supplemented with the metalloprotease inhibitor EDTA and addition of equivalent amounts of the catalytical domain of MMP-3 (cd-MMP-3), used to activate MMP-9. In addition, the highest concentration of MMP-9 was also incubated separately in assay buffer as a control for non-substrate fragment bands. Molar enzyme : substrate (E : S) ratios ranged between (1 : 1250 to 1 : 10) for tubulin, Jo-1 and citrate synthase, and between (1 : 6250 to 1 : 10) for skeletal muscle actin. For the Arp2/3 complex mass E : S ratios were (1 : 625 to 1 : 5). The duration of incubation was 4.5 h for tubulin cleavage, 18 h for proteolysis of Jo-1, the Arp2/3 complex and citrate synthase, and 24 h for actin. SDS-PAGE analysis shows concentration-dependent degradation of the substrates by MMP-9 and generation of multiple fragments (*). Visualization of the substrate before incubation is indicated by t0. Apparent molecular masses are shown in Da at the right side of the gels.

with the actin meshwork and/or with microtubuli (Table 4). About 40% of the identified candidate substrates are components of the ICM or associated with ICM proteins. 30% of the substrates are connected with the actin cytoskeleton, whereas 17% show association with microtubuli.

Multiple intracellular MMP-9 candidate substrates are described autoantigens

To integrate the results in a broad immunological context, a literature search was conducted to determine if the list of MMP-9 candidate substrates (Table 1) contained known autoantigenic molecules. Candidate substrates that have been described as autoantigenic molecules and the pathology in which they were discovered are listed in Table 5. Interestingly, autoantibodies against 61% of the discovered intracellular MMP-9 candidate substrates have been described in pathologic conditions. About 50% of the candidate substrates are autoantigens in one or more autoimmune conditions, whereas 30% of the intracellular MMP-9 substrates are known cancer-associated antigens. Many candidate substrates correspond to autoantigens in multiple conditions. 32% of the potential substrates are autoantigens in the prototypic systemic autoimmune condition systemic lupus erythematosus (SLE). Known prevalences (%) of these autoantigens in SLE were added to Table 5. Furthermore, 16% of the identified substrates are autoantigens in systemic sclerosis, 17% in Sjögren's syndrome, 27% in rheumatoid arthritis, 20% in inflammatory bowel disease and 20% in various (autoimmune) liver diseases. In conclusion, proteolysis of intracellular autoantigens by MMP-9 or other inflammation-associated proteases may affect multiple (systemic) pathologies.

Discussion

Matrix metalloproteinases (MMPs) are notoriously known for their ECM remodeling functions as well as for their regulatory 'processing' of cytokines, protein hormones, chemokines and cell membrane-bound molecules.[1,2] However, the intracellular

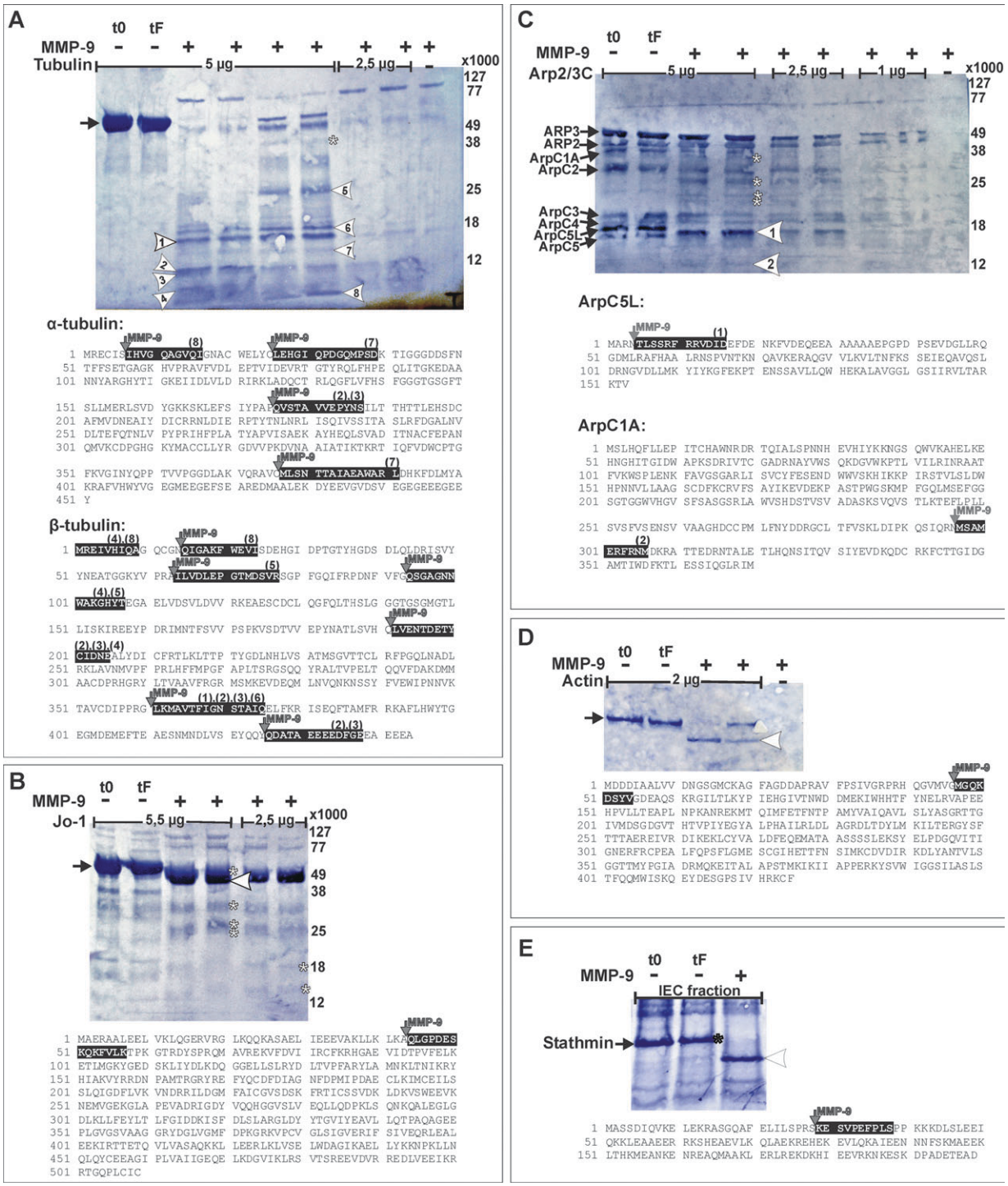

Fig. 4 *Identification of the MMP-9 cleavage sites by Edman sequencing*. Recombinant/purified proteins were incubated in the absence (tF, −) or presence (+) of various concentrations of activated MMP-9. The highest MMP-9 concentration was also incubated separately in assay buffer as a control for non-substrate fragment bands. The incubated samples were transferred to PVDF by electroblotting and novel NH_2-termini of fragment bands were sequenced by Edman degradation. Identified NH_2-terminal sequences are indicated on a black background together with the MMP-9 cleavage sites (grey arrows). When sequences were detected in multiple fragment bands (white arrowheads on the blots), the numbers of these fragments were added between brackets above the black background of the sequenced stretch. Protein bands that were resistant to Edman sequencing are marked with an asterisk (*). The intact substrate is indicated by a black arrow. Visualization of the substrate before incubation is indicated by t0. Apparent molecular masses are shown in Da at the right side of the gels. Molar enzyme : substrate ratios and incubation intervals were (1 : 50 to 1 : 5) and 4.5 h for tubulin (A); (1 : 10 to 1 : 2) and 18 h for Jo-1 (B); mass E : S (1 : 5 to 1 : 1) and 18 h for the Arp2/3 complex (C), and (1 : 20 to 1 : 2) and 24 h for non-muscle platelet actin (D). Stathmin (E) was the only substrate to be identified by Edman degradation after blotting of the ion exchange (IEC) fraction. Although the intact protein was resistant to Edman degradation, the NH_2-terminal fragment that misses the first 28 amino acids could be sequenced.

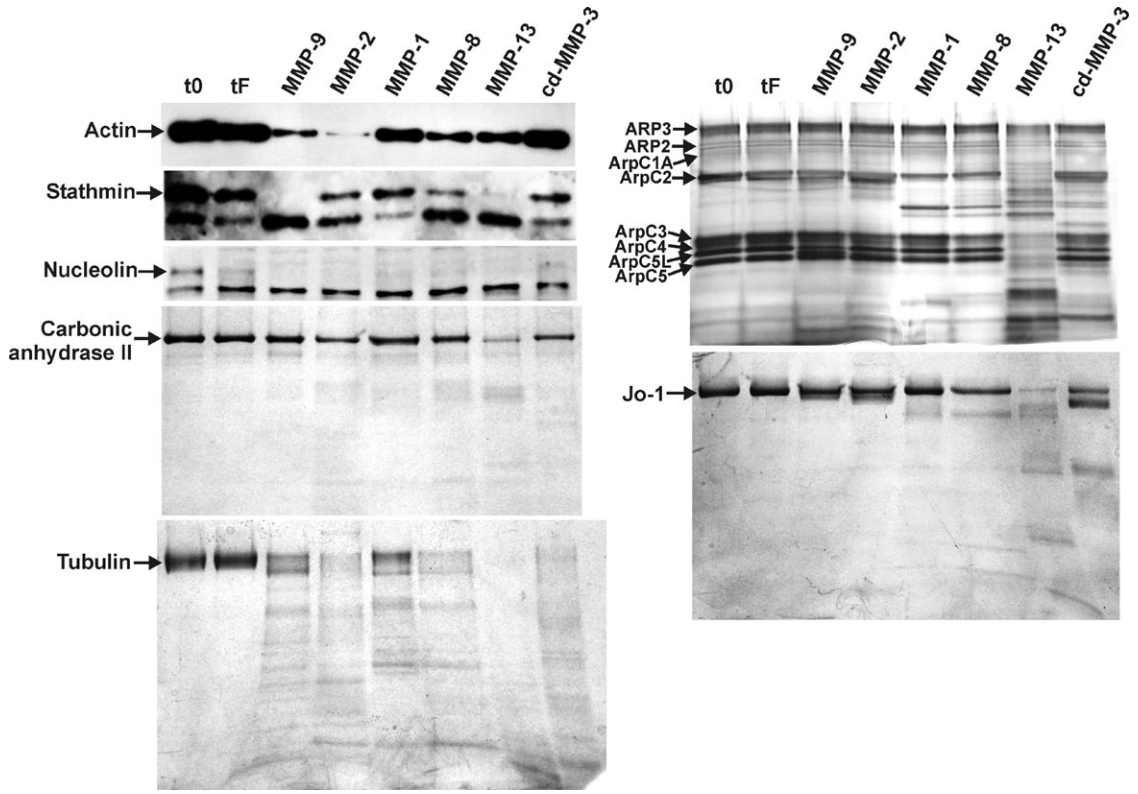

Fig. 5 *Proteolysis of novel intracellular MMP-9 substrates by various MMPs.* THP-1 cytosol and the purified or recombinant substrates were incubated without (tF) or in the presence of active MMP-9, -2, -1, -8, -13 and the catalytical domain of MMP-3 (cd-MMP-3). THP-1 cytosol was incubated for 4.5 h and MMP concentrations were 100 nM for actin and stathmin, and 5 nM for nucleolin. Molar enzyme : substrate (E : S) ratios were (1 : 20) for tubulin, and Jo-1, and (1 : 30) for carbonic anhydrase II. Mass E : S for the Arp2/3 complex was (1 : 2.7). The substrates were incubated for 18 h, except for tubulin (4.5 h). SDS-PAGE and/or Western blot analysis of these digestions show a comparison of the proteolysis of the intracellular substrates by the MMPs tested. Analysis of the substrate before incubation is marked as t0.

degradome of MMPs still remains relatively unexplored.[219] In our previous work we showed that gelatinase B/MMP-9 cleaves multiple intracellular protein substrates released during cellular necrosis of human myelomonocytic THP-1 cells.[6] In order to further characterize the intracellular degradome of MMP-9, multidimensional degradomic approaches were developed and applied to the cytosolic fraction of THP-1 cells. By the application of both high-throughput methods termed '2D-degradomics' and 'fragment degradomics', we were able to isolate 100–200 candidate substrates of which a selection was isolated and subjected to MS/MS. Of this selection, about 70 intracellular MMP-9 candidate substrates were identified. The intracellular MMP-9 substrate CAP1, discovered by our one-dimensional degradomics approach,[6] was corroborated with both new approaches. In addition, about 25% of the defined substrates were identified previously as MMP-9 candidate substrates by diverse proteomic approaches, ranging from two-dimensional gel electrophoresis to a label-free ultra performance liquid chromatography (UPLC)-based approach.[3,4,20,21] Biochemically or *in vivo* confirmed MMP substrates are considered to be high-confidence MMP-9 candidate substrates. Potential substrates that were identified both as a substrate and as a fragment or corroborated by identification in many different IEC fractions, also represent high-confidence candidate substrates. A selection of both strong and low confidence candidates (single identification) was subjected to *in vitro* cleavage of the recombinant or purified substrate, or to substrate-specific Western blot analysis of MMP-9-digested and intact cytosol (or IEC fractions) for confirmation. In this way, the tested high-confidence (α-, β- and γ-actin, HMGB1, stathmin) and low-confidence (α- and β-tubulin, annexin I, nucleolin, Arp2/3 complex subunits, Jo-1, citrate synthase, carbonic anhydrase II) candidates were confirmed as novel intracellular MMP-9 substrates. The only exception was carbonic anhydrase II, whose original cleavage might have been indirect *e.g.* by an MMP-9-activated protease. However, carbonic anhydrase II was clearly degraded by MMP-13 (Fig. 5). MMP-13 was most efficient at the cleavage of several tested intracellular substrates. Interestingly, a recent study found activated MMP-13 in the nucleus of neural cells after cerebral ischemia,[220] identifying MMP-13 as a probable candidate for intracellular proteolytic actions. These biochemical confirmations point to the robustness of our multidimensional degradomics approaches, and also highlight the need for confirmation of proteomic results by complementary methods. Besides the high validity of the identifications, these unprejudiced, systematic approaches have a number of advantages in comparison with other current methods. First, the substantial loading capacity of ion exchange columns makes it possible to start with a high protein amount. This, together with the concentration of the ion exchange fractions in 2D-degradomics,

allowed us to identify low abundancy class substrates that might have been missed starting from a small unfractionated protein sample. HMGB1, HMGB2, hepatoma-derived growth factor, nitrilase homolog 2, positive cofactor 4 and c-myc-responsive protein Rcl are examples of low abundancy class molecules identified in our screening. Second, by incubating fractionated cytosol with MMP-9, less bias occurs for highly abundant, high-affinity substrates that 'consume' the protease in a complex mixture and mask lower-affinity, but still potential physiological substrates. Indeed, HMGB1 was not cleaved in unfractionated cytosol in the presence of highly abundant substrates such as actin, tubulin and CAP1. However, cleavage by MMP-9 was clearly detected in the corresponding ion exchange fraction and may be of physiological interest during necrosis (*vide infra*). In addition, 2D-degradomics, with fractionation before MMP-9 cleavage, yields 10–20 times more identifications in comparison with cleavage of the total protein pool in fragment degradomics. Third, as these straightforward degradomic approaches do not require complex and expensive equipment, the application of this technology is accessible to every laboratory with interest in protease degradomics. Fourth, although these methods only yield *in vitro* substrates, the extensive and unbiased list of candidates generated by this technology may reveal unexpected target proteins that can be further validated in the corresponding *in vivo* disease models. Finally, multidimensional degradomics can be extended to other cell types, culture media, tissues or body fluids, to various time points or concentrations, and to the use of additional MMPs, proteases and protease inhibitors, turning it into a novel powerful tool for the mapping of the intracellular MMP degradome, as well as for high-throughput identification of new protease substrates.

Remarkably, about 40% of the identified candidate substrates are components of the intracellular matrix (ICM) or associated with ICM proteins. Identification of less abundant substrates, for example by the use of the more sensitive silver stain technique, or by the application of other more sensitive technology may lower this 40% of ICM molecules in the substrate list. However, the fact that so many ICM molecules are degraded by MMP-9 and other MMPs is noteworthy. 30% of the potential substrates are connected with the actin cytoskeleton, whereas 17% of the candidates show association with microtubuli. Both actin and tubulin proteins were shown to be degraded into multiple fragments. This suggests that MMP-9 may facilitate extracellular clearance of these highly abundant proteins. The major cleavage of actin by MMP-9 at the NH_2-terminus between Gly46 and Met47 may suppress actin polymerization as the actin NH_2-terminus (Asp2-Val43) contains one of the sites for protein-protein interaction that are involved in the actin filament formation.[221,222] Indeed, actin truncated by neutrophil elastase between Val43-Met44 was no longer able to polymerize.[223] Besides a binding site for actin-binding proteins that regulate actin function and polymerisation,[224,225] the actin NH_2-terminus contains the DNaseI-binding loop of actin (residues 38-52).[222] Proteolytic modifications of loop 38-52 by subtulisin, chymotrypsin and other proteases have been shown to perturb or abolish salt-induced polymerization and the DNaseI-inhibitory capacity of actin.[226–230] In addition, the NH_2-terminal polypeptide cleaved off by neutrophil elastase (residues 2–43) suppresses neutrophil motility and chemotaxis, as well as elastase production by stimulated neutrophils.[223] Integration of this knowledge leads to the conclusion that the major MMP-9 cleavage of actin at Gly46-Met47 has profound effects on physiological and pathological actin functions.

The other main cytoskeleton component is the microtubule-building block, tubulin, which is composed of a heterodimer of α- and β-tubulin, both of which were shown to be extensively cleaved by MMP-9. The acidic COOH-terminal tails of α- and β-subunits have considerable functional significance as they form the docking site for many microtubule-regulating proteins and are the subject of most post-translational modifications that regulate actin function.[231] Removal of the COOH-terminal ends of both subunits by subtulisin lowers the critical concentration for polymerization about 50-fold.[232] Likewise, proteolysis of α-tubulin by granzyme B (at Asp438-Ser439) and by granzyme M (at Leu269-Ala270) both significantly enhance polymerization rates *in vitro* and induce microtubule network disorganization in tumor cells *in vivo*, which likely contributes to cell death.[233–235] β-Tubulin is not cleaved by granzyme A and B, but by granzyme K (at Arg62-Ala63 and Arg282-Ala283).[236] In addition, COOH-terminal truncations of β-tubulin lacking Ala428 yield tubulins which are not compatible with microtubule formation.[237] As MMP-9 removes the acidic tails in both α- and β-subunits, including Ala428 in β-tubulin, it is very likely that proteolytic modification of tubulin by MMP-9 perturbs or abolishes tubulin functions.

Besides the major cytoskeleton constituents, actin and tubulin, many high-confidence or confirmed MMP-9 targets are cytoskeleton-associated proteins which regulate actin and microtubule polymerization and function. Degradation of these essential actin/tubulin-binding proteins may avert branching and polymerization in the extracellular space, thus preventing concomitant extracellular cytoskeleton toxicity. The actin-related protein-2/3 (Arp2/3) complex has a crucial role in the formation of branched-actin-filament networks during diverse processes ranging from cell motility to phagocytosis.[23] CAP1 promotes rapid actin turnover and is important for cell morphology, migration and endocytosis.[26,27] Ezrin and moesin are members of the *E*zrin/*r*adixin/*m*oesin (ERM) family of proteins, which have been reported to function as cross-linkers between the plasma membrane and the cortical cytoskeleton.[30] Filamin-B is one of the filamins, which can crosslink actin filaments to form orthogonal networks and connect membrane proteins to the actin cytoskeleton.[32] Gelsolin is an actin-filament severing and capping protein involved in cell motility, phagocytosis and apoptosis.[33] In addition to this cytoplasmic regulator of actin organization, the gelsolin gene expresses a splice variant coding for a distinct isoform, plasma gelsolin, which is secreted into extracellular fluids. The secreted form of gelsolin has been implicated in a number of processes such as the extracellular actin-scavenging system and the presentation of lysophosphatidic acid and other inflammatory mediators to their receptors.[238] The extracellular actin-scavenging system in the vascular compartment involves 2 proteins: gelsolin that releases monomers from the toxic filaments, and Gc-globulin,

Table 4 ICM-associated MMP-9 (candidate) substrates

Substrate	Cytoskeleton association		Ref(s).
	Actin	Microtubuli	
α-Actin β-Actin γ-Actin	Yes	No	22
Actin-related protein 2/3 complex	Yes	No	23
Apoptosis-linked-gene-2-interacting-protein X	Yes	No	24
Annexin I	Yes	No	25
Adenylyl cyclase-associated protein-1	Yes	No	26, 27
Actin regulatory protein CAP-G	Yes	No	28
Cyclophilin A	No	Yes	29
Ezrin	Yes	No	30
Fascin	Yes	No	31
Filamin B	Yes	No	32
Gelsolin	Yes	No	33
Heat shock protein 27	Yes	Yes	34, 35
Heat shock cognate protein 70	Yes	Yes	34
Heat shock protein 90α/β	Yes	Yes	34, 36
IQ motif containing GTPase activating protein 1	Yes	Yes	37
Microtubule-associated protein RP	No	Yes	38
Moesin	Yes	No	30
Nucleolin	Yes	No	39
Nucleoside diphosphate kinase	No	Yes	40
Stathmin	No	Yes	41
Tubulin-specific chaperone A	No	Yes	42
α-Tubulin β-Tubulin	No	Yes	43

which complexes the freed monomers. Both actin-gelsolin and actin-Gc-globulin complexes are subsequently cleared by the hepatic phagocytosis system much more efficiently than free proteins (half-lives of 30 min *vs.* 1–2 days, respectively). Nevertheless, excessive release of cellular actins or decreased activity of the actin-scavenger system causes severe pathological conditions such as hepatic necrosis, adult respiratory distress syndrome (ARDS), septic shock, and complications of pregnancy, as discussed previously.[8–12] Plasma gelsolin has been shown to be cut into several fragments by various MMPs, including MMP-9, which resulted in considerable loss of its depolymerizing activity.[3,4] This suggests that MMPs may weaken the extracellular actin-scavenging system by cleaving gelsolin, culminating in or enhancing pathological conditions induced by extracellular actin. Conversely, as MMP-9 and other MMPs degrade actin, they may contribute to actin clearance and scavenging. Hence, it is a great challenge to elucidate whether the sum of the proteolytic activities present at the site of injury enhance or, on the contrary, decrease clearance of extracellular actin.

Among the microtubule-binding proteins, stathmin was shown to be a key regulator of microtubule dynamics. Stathmin causes depolymerization of microtubule filaments into small tubulin oligomers and inhibits polymerization of tubulin monomers. This activity is inhibited by reversible phosphorylation of four serine residues (Ser16, Ser25, Ser38, Ser63).[41,239] Proteolysis of stathmin by MMP-9 between Ser28 and Lys29 removes two of these regulatory phosphorylation sites as well as a tubulin binding site. In addition, the NH_2-terminal capping domain, which inhibits longitudinal protofilament contacts,[239] is disrupted by MMP-9. As a consequence, disturbing stathmin actions may be an additional effect of MMP-9 on tubulin function. In conclusion, unveiling the biological significance of ICM protein proteolysis by MMPs and its consequences on inflammation and extracellular actin toxicity and immunogenicity after tissue injury, may attribute novel (immuno)regulatory properties to MMP-9 and other ICM-degrading proteases.

The role of gelatinase B/MMP-9 in the pathogenesis of organ-specific autoimmune diseases has been well established, *e.g.* generating remnant epitopes of myelin basic protein,[15] interferon-β[240] or αB-crystallin[241] in multiple sclerosis, of insulin in diabetes[16] and of collagen type II in rheumatoid arthritis.[14] Most of these substrates are naturally occurring in the extracellular milieu. One of the goals of the present study was to examine systemic autoimmune antigens, which most often are ubiquitous intracellular proteins. We found that autoantibodies against about 2/3 of the discovered intracellular MMP-9 candidate substrates have been described in multiple pathologies: 50% of the candidate substrates were found to be autoantigenic targets in (an) autoimmune condition(s), whereas 30% of the intracellular MMP-9 substrates were described as cancer-associated antigens. Systemic autoimmune diseases are a genetically complex heterogenous group of diseases in which the immune system targets a diverse, but highly specific group of ubiquitous intracellular autoantigens. It has not been fully clarified how these normally sequestered molecules are rendered capable of sustaining a prolonged autoimmune response. However, increasing evidence points to a tolerance-breaking role for cell death abnormalities, such as increased rates of apoptosis and/or defective clearance of apoptotic cells.[242,243] Indeed, in systemic autoimmune diseases associated with impaired clearance of apoptotic cells, the unphagocytosed apoptotic cell load may undergo secondary necrosis, exposing intracellular proteins to cell death-associated post-translational modifications. These modifications may enhance autoantigen immunogenicity by presenting novel or cryptic self epitopes to the immune system for which tolerance has not been established. Repeated stimulation by these neo-epitopes could induce and sustain an autoantibody response.[244–247] Autoantigen proteolysis is one of the major post-translational modifications generating neo-epitopes.[7,244,248] Interestingly, Casiano and co-workers showed that distinct autoantigen cleavage products are generated during necrosis *vs.* apoptosis, probably by different proteases, as the cleavages were caspase-independent *vs.* caspase-dependent, respectively.[249] In addition, the progression from apoptosis to secondary necrosis was also shown to be associated with additional proteolysis of specific autoantigens in a caspase-independent manner.[250] As MMP-9 was shown to proteolyse intracellular proteins released from necrotic cells,[6] it would be tempting to speculate that MMPs may contribute to these necrosis-associated caspase-independent cleavages, triggering or stimulating systemic autoimmunity. For example, cleavage of the autoantigen Jo-1 or histidyl-tRNA synthetase by MMP-9 between the residues Ala43 and Gln44 releases the Jo-1 NH_2-terminus which contains the major immunodominant epitope(s).[251–253] In addition, both intact and granzyme B-cleaved Jo-1 (residues 1–48) can recruit $CD4^+$ and $CD8^+$ T cells, immature

Table 5 Autoantigenic MMP-9 (candidate) substrates

Substrate	Disease (autoantigen %)[a]	Ref(s).
α-Actin β-Actin γ-Actin	SLE (9–26%), systemic sclerosis, autoimmune and chronic hepatitis, various liver disorders, coeliac disease (90%), Sjögren's syndrome, rheumatoid arthritis, idiopathic nephrotic syndrome, autoimmune inner ear disease, autoimmune neutropenia, Crohn's disease, ulcerative colitis, cardiovascular diseases, acute leukemia, EAU, visceral leishmaniasis	44–66
Aldolase A	Rheumatoid arthritis, diabetic retinopathy, Alzheimer's disease, chronic myloid leukemia, acute and chronic hepatitis	67–71
Annexin I	SLE (23–32%), rheumatoid arthritis, dermatomyositis, psoriasis, inflammatory bowel disease, immunoinfertility, lung cancer, asthma	55, 72–80
Adenylyl cyclase-associated protein-1	SLE, rheumatoid arthritis	55, 81, 82
Carbonic anhydrase II	SLE (24–32%), systemic sclerosis, Sjögren's syndrome, polymyositis, type 1 diabetes, primary biliary cirrhosis, ulcerative colitis, Graves' disease, autoimmune pancreatitis, (autoimmune) liver disease, endometriosis	55, 83–91
Citrate synthase	SLE, rheumatoid arthritis, systemic sclerosis, undifferentiated connective tissue disease, polymyositis/dermatomyositis, Raynaud syndrome, Sjögren's syndrome, primary biliary cirrhosis, allograft vasculopathy	92–94
Cyclophilin A	SLE, Lyme disease	95, 96
Elongation factor 1-α1	SLE, rheumatoid arthritis, myositis, Q fever, atopic dermatitis	55, 81, 82, 97–99
Elongation factor 2	SLE	100
α-Enolase	SLE (24–27%), systemic sclerosis, mixed cryoglobulinemia, Hashimoto's encephalopathy, rheumatoid arthritis, Behçet's disease, Kawasaki disease, relapsing polychondritis, acute and chronic myloid leukemia, opsoclonus myclonus syndrome, autoimmune and cancer-associated retinopathy, multiple sclerosis, lung cancer, premature ovarian failure, ''wet'' age-related macular degeneration, severe asthma, pituitary diseases, post-streptococcal autoimmune CNS disease, autoimmune liver disease, membranous nephropathy, inflammatory bowel disease, endometriosis, vasculitis, coeliac disease	55, 65, 66, 71, 82, 101–122
Eukaryotic translation initiation factor 5A	Ovarian cancer	123
Ezrin	Rheumatoid arthritis	124
Ferritin light chain	Rheumatoid arthritis	125
Filamin B	Experimental lupus nephritis, myasthenia gravis, chronic lymphocytic leukemia	126–128
β-glucuronidase	Wegener's granulomatosis, polyarteritis, Churg Strauss syndrome, glomerulonephritis, inflammatory bowel disease	129–131
Glutathione S-transferase P	Autoimmune hepatitis, glaucoma	132, 133
Hepatoma-derived growth factor	Ulcerative colitis	134
Histidyl-tRNA synthetase	Anti-synthetase syndrome (60–80%), myositis, SLE, rheumatoid arthritis, Sjögren's syndrome, cutaneous lupus, Sharp's syndrome, Crohn's disease, cutaneous vasculitis, cirrhosis	97, 135, 136
High-mobility group protein B1 High-mobility group protein B2	SLE (HMGB1 37%; HMGB2 18%), rheumatoid arthritis, drug-induced lupus, Sjögren's syndrome, systemic sclerosis, autoimmune hepatitis, primary biliary cirrhosis, chronic hepatitis B and C, ulcerative colitis	55, 137–143
Heat shock protein 27	Guillain–Barré syndrome, Graves' disease, immunoinfertility, glaucoma, gynecologic cancer	35, 72, 144–147
Heat shock cognate protein 70	SLE (35%), rheumatoid arthritis (5%), psoriatic arthritis (5%), juvenile dermatomyositis, chronic hepatitis, Guillain–Barré syndrome, immune thrombocytopenic purpura, type I diabetes, mixed connective tissue disease, localized scleroderma, systemic sclerosis, autoimmune liver diseases, multiple sclerosis, chronic myeloid leukemia, glaucoma, autoimmune hearing loss, atherosclerosis, asthma, graft-versus-host-disease, heat-induced diseases, malaria, Menière's disease, cancer-associated retinopathy, Behçet's disease, sarcoidosis, pars planitis, Vogt–Koyanagi–Harada disease, autoimmune thyroiditis, Crohn's disease (8–14%), ulcerative colitis (23%), Sjögren's syndrome (0–8%), dilated cardiomyopathy, Parkinson's disease, schizophrenia, hepatocellular carcinoma, ESCC, experimental autoimmune orchitis	48, 71, 109, 146, 148–173
Heat shock protein 90α/β	SLE (25–50%), multiple sclerosis, Guillain–Barré syndrome, Crohn's disease (4%), ulcerative colitis (0–8%), Sjögren's syndrome (0–4%), acute mania, ovarian cancer, breast cancer, autism, schizophrenia, graft-versus-host-disease, heat-induced diseases, malaria, osteosarcoma	55, 146, 149, 157–159, 171, 174–182
IQ motif containing GTPase activating protein 1	Ovarian cancer, bullous skin disease	174, 183
Moesin	Acquired aplastic anemia, rheumatoid arthritis	124, 184
Nucleolin	SLE (17–64%), rheumatoid arthritis, Sjögren's syndrome, systemic sclerosis, polymyositis, polymyalgia rheumatica, acute hepatitis A, infectious mononucleosis, scleroderma-like graft-versus-host-disease	55, 185–187
Nucleosome assembly protein 1-like 1	Colon adenocarcinoma	188

Table 5 (*continued*)

Substrate	Disease (autoantigen %)[a]	Ref(s).
Nucleoside diphosphate kinase	Hepatocellular carcinoma	189
Oxygen-regulated protein 150	Type I diabetes	190
Peroxiredoxin 2	Breast carcinoma, immunoinfertility	72, 191
Peroxiredoxin 6	ESCC, periodontitis	192, 193
Phosphoglycerate kinase 1	SLE, experimental autoimmune orchitis	170, 194
Rho GDP dissociation inhibitor (GDI) β	Acute leukemia	65
Small nuclear ribonucleoprotein Sm D3	SLE (10–55%), Sjögren's syndrome, Chagas' disease, tuberculosis, lymphoma	55, 195–200
SUMO-1 activating enzyme subunit 2	Dermatomyositis	201
Threonyl-tRNA synthetase	Anti-synthetase syndrome (5–15%), systemic sclerosis, polyarthritis, sclerodactyly	97, 202
α-Tubulin β-Tubulin	SLE, rheumatoid arthritis, multiple sclerosis, demyelinating polyneuropathies, autoimmune thyroid diseases, Guillain–Barré syndrome, LHON, type I diabetes, relapsing polychondritis, chronic myloid leukemia, Sydenham's chorea, allergic rhinitis, autoimmune hearing loss, nasopharyngeal carcinoma, hepatocellular carcinoma, neuroblastoma, parasitic infections, acute leukemia, breast carcinoma, EAU	49, 54, 55, 60, 64, 65, 71, 112, 189, 191, 203–218

[a] CNS, central nervous system; EAU, experimental autoimmune uveoretinitis; ESCC, esophageal squamous cell carcinoma; LHON, Leber's hereditary optic neuropathy; SLE, systemic lupus erythematosus. Known prevalences (%) of the autoantigen in a particular disease are shown in parentheses.

dendritic cells and activated monocytes, whereas an NH_2-terminal deletion mutant has no such 'recruiting' activity.[254] Remarkably, a unique form of Jo-1 exists in the lung that is highly susceptible to cleavage by granzyme B and may be associated with the onset of anti-Jo-1 myositis-interstitial lung disease overlap syndrome. Since MMP-9 also releases the Jo-1 NH_2-terminus (residues 1–43) this cleavage may contribute to the onset and propagation of anti-Jo-1 autoimmunity in myositis.

Auto-antibodies to both annexin I and nucleolin are found with high incidence in systemic lupus erythematosus (references in Table 5) and are degraded very efficiently by MMP-9. Annexin I is a Ca^{2+}/lipid-binding protein with anti-inflammatory properties in its NH_2-terminus. Annexin I reduces neutrophil adhesion and emigration to inflamed vessels and stimulates neutrophil apoptosis, possibly preventing further tissue damage after inflammation.[255,256] These anti-inflammatory actions of annexin I may be terminated by proteolytic degradation. Indeed, a 33 kDa truncation product was found in bronchoalveolar lavage fluid from patients with cystic fibrosis. Annexin I appeared to be truncated by neutrophil elastase at the NH_2-terminal portion, resulting in greatly diminished functional activity.[257] Annexin I was cleaved in a similar way by neutrophil proteinase 3. This may be one of the proteinase 3 actions underlying its pro-inflammatory phenotype, that is, removal of the neutrophil-inhibitory properties of annexin I.[258] Interestingly, annexin I co-localizes with MMP-9 in the gelatinase granules of neutrophils.[259] In addition, MMP-9 also generates a 33 kDa fragment from annexin I (Table 1).

High mobility group box 1 protein (HMGB1) is a non-histone nuclear protein with a dual function. Inside the cell, it binds DNA and regulates a variety of processes such as transcription. During primary and secondary necrosis—but not apoptosis—HMGB1 is passively released from the cell and can function as an alarmin or 'damage-associated molecular pattern' (DAMP)[260] to alert the immune system after tissue damage.[261] As an alarmin, HMGB1 may play an important pro-inflammatory role in the pathogenesis of multiple rheumatic diseases.[262] Development of autoantibodies to HMGB1 is a common feature of many autoimmune disorders (*cf.* Table 5). Increased levels of HMGB1 are found in the sera of mice and patients with SLE.[263] In addition, HMGB1-nucleosome complexes detected in plasma from SLE patients induce maturation of antigen-presenting cells, which may be crucial for breaking the immunological tolerance against nucleosomes/dsDNA.[264] Thrombomodulin, an endothelial thrombin-binding protein, binds HMGB1 and enhances thrombin-mediated HMGB1 cleavage decreasing the proinflammatory activity of HMGB1.[265] In the light of these major pro-inflammatory and 'adjuvant' roles of HMGB1, it would be exciting to examine whether HMGB1 cleavage by MMP-9 decreases or enhances its pathogenic effects in systemic autoimmune diseases. In conclusion, investigating the effect of proteolysis of intracellular autoantigens by MMP(-9) on their immunogenic potential may yield novel insights in the roles of MMPs in systemic autoimmunity.

Proteolysis is a key regulatory process that promotes the (in)activation, translocation, and/or degradation of proteins. As a consequence, the development of novel degradomics tools is a booming field.[21,266–269] By the application of multidimensional degradomics we started an unbiased exploration of the proteolytic modification of intracellular proteins. Identification of the intracellular MMP-9 substrate repertoire and characterization of the cleavage sites are essential for understanding the biological functions of intracellular protein processing by MMP-9 and other proteases. Extracellular proteolysis may be crucial to remove abundant ICM proteins, in this way avoiding actin toxicity and immunogenicity after extensive necrosis, and dampening inflammation after tissue injury. In accordance with the immunogenetic make-up of an individual, proteolytic modification of systemic

autoantigens may contribute to the generation of immunodominant 'neo-epitopes', initiating or sustaining systemic autoimmunity. The further study of the physiological and pathological consequences of proteolysis of ICM molecules, autoantigens, alarmins and other key molecules may result in the discovery of novel roles for proteolytic modification and yield surprising immunologic and therapeutic insights. In this way, our *in vitro* findings might be corroborated or challenged by future data from *in vivo* experiments.

Materials and methods

Substrates and antibodies

Recombinant human Jo-1 was purchased from Diarect (Freiburg, Germany). Porcine citrate synthase, bovine carbonic anhydrase II and rabbit skeletal muscle actin were from Sigma (St. Louis, MO, USA). Human skeletal actin, human non-muscle platelet actin, bovine brain tubulin and the bovine brain Arp2/3 complex were purchased from Cytoskeleton (Denver, CO, USA). Rabbit polyclonal anti-annexin I antibody (H-65) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal anti-nucleolin, anti-HMGB1 and anti-actin antibodies were from Sigma. Rabbit polyclonal anti-stathmin antibody was from Calbiochem (Darmstadt, Germany).

Preparation of THP-1 cytosol

The human myelomonocytic cell line THP-1[270] was cultured in RPMI 1640 medium (Bio Whittaker Europe, Verviers, Belgium), supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, California, USA) and 1.5% sodium bicarbonate. At the end of the log phase, cells were collected by centrifugation, washed 3-4 times with phosphate-buffered saline (PBS) or physiological assay buffer (20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 10 mM $CaCl_2$) and cell pellets were stored at −20 °C. For ion exchange chromatography, the cell extracts were resuspended in 20 mM ethanolamine pH 9.5 supplemented with a general proteinase inhibitor cocktail (PI, Complete Mini EDTA-free, Roche, Basel, Switzerland) (1 tablet/10 mL). Alternatively, for cleavage assays the cell extracts were diluted in assay buffer (100 mM Tris/HCl, pH 7.4, 100 mM NaCl, 10 mM $CaCl_2$) supplemented with 0.01% Tween-20 (Applichem Inc., Cheshire, CT, USA) and PI. The cells were lysed by 3–4 freeze–thaw cycles and DNA was sheared by sonication. The membrane fraction was removed by ultracentrifugation at 110 000 g with a swing-out rotor SW50.1 in a Beckman L7-55 Ultracentrifuge (Beckman Coulter, Fullerton, CA, USA) during 75 min at 4 °C. The resulting supernatant cytosol was kept at −20 °C for further analysis.

Ion exchange chromatography (IEC)

THP-1 cytosol in ethanolamine buffer (pH 9.2) was filtrated (0.22 μm) and applied to a 1 mL cation exchange column (S1-Uno, Bio-Rad, Hercules, CA, USA). Proteins were eluted with a 40 mL linear gradient from 0 M to 0.5 M NaCl, followed by a 10 mL linear gradient from 0.5 to 1 M NaCl. Elution fractions were neutralized with 1M Hepes, pH 7.45 and kept at −20 °C for further analysis, whereas flow through fractions were pooled and loaded onto a 1 mL anion exchange column (Q1-Uno, Bio-Rad). Elution was carried out with a 40 mL linear gradient from 0 to 0.5 M NaCl, followed by a 10 mL linear gradient from 0.5 to 1 M NaCl. Eluted factions were neutralized with 1M Hepes, pH 7.45.

Purification and activation of recombinant human MMPs

Recombinant human MMP-9 was expressed in Sf9 insect cells and purified by gelatin-Sepharose affinity chromatography as described previously.[271,272] Purified pro-MMP-9 (10 μM) was activated with 0.1 μM of the catalytic domain of human MMP-3 (cd-MMP-3, Calbiochem) for 1.5 h at 37 °C in assay buffer supplemented with 0.001% Tween-20 (Sigma). MMP-1, MMP-8 and MMP-13 were activated with APMA (*p*-aminophenylmercuric acetate) according to the instructions of the manufacturer (R&D Systems, Abingdom, UK). The commercial preparation of MMP-2 (R&D Systems) was found to be active without activation and therefore used as such.

Concentration and incubation of the IEC fractions

Cation and anion exchange (CEC and AEC) elution fractions were dialyzed against salt-free assay buffer (100 mM Tris/HCl, pH 7.4, 10 mM $CaCl_2$) supplemented with 0.01% Tween-20 and concentrated 2–10 times on micro-spin columns with a 10 kDa cut-off membrane (Microcon YM-10, Millipore Corporation, Billerica, MA, USA). Before and after concentration, protein concentrations of the fractions were determined with the Bradford protein assay (Bio-Rad) as described.[273] Concentrated fractions were incubated in the absence or presence of 100 nM activated MMP-9 for 24 h at 37 °C. For fragment degradomics, pooled CEC flow through was neutralized and incubated in the absence or presence of 100 nM activated MMP-9 for 24 h at 37 °C. Fragments generated by incubation were enriched by filtration through a 50 kDa limitating membrane (Microcon YM-50, Millipore Corporation). Flow through fractions of the 50 kDa microspin columns were dialyzed against salt-free assay buffer and further concentrated on a 3 kDa membrane (Microcon YM-3, Millipore Corporation).

In vitro cleavage assays

THP-1 cytosol and recombinant or purified proteins were incubated for the indicated time intervals in the absence or presence of activated MMP-9, cd-MMP-3, 40 nM activated MMP-9 supplemented with 10 mM of the metalloprotease inhibitors 1,10-*o*-phenanthroline or EDTA and with active MMP-1, -2, -8, -13 (5 nM or 100 nM). As a control for non-substrate fragments, the highest concentration of activated MMP-9 was incubated separately in assay buffer without addition of substrate. The corresponding molar enzyme : substrate ratios can be found in the figure legends. Incubations were stopped with 2× Laemmli sample buffer (0.125 M Tris/HCl, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.05% bromophenol blue).

SDS-PAGE and Western blot analysis

Incubated fractions and digested recombinant or purified proteins were analyzed using SDS-PAGE with Tris-tricine as

running buffer (10%, 14% or 18% Prosieve® 50 Gel Solution, Lonza, Basel, Switzerland) and visualized with Coomassie Brilliant Blue R-250 (Sigma) or by silver staining analysis (Silverquest™ Silver Staining kit, Invitrogen) for actin. After electrophoresis, the proteins were transferred from the gels to PVDF (0.45 μm, Applied Biosystems, Foster City, CA, USA) or nitrocellulose membranes (0.2 μm, Whatman, Maidstone, UK) by semi-dry or wet electroblotting. The membranes were blocked with 5% non-fat dry milk (Bio-Rad) or 2% ECL advance blocking reagent (GE Healthcare) in Tris-buffered saline (20 mM Tris/HCl, pH 7.6, 137 mM NaCl) containing 0.1% Tween-20 and then probed with a substrate-specific primary antibody. After washing, the membrane was incubated with the appropriate peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA). Finally, proteins were detected according to the manufacturers recommendations of the ECL plus™ chemiluminescence kit (GE Healthcare).

Protein identification by tandem mass spectrometry (MS/MS)

For MS/MS, proteins were stained with Coomassie Brilliant Blue R-250 after SDS-PAGE analysis and the protein band(s) of interest were excised from the gel and identified by matrix-assisted laser desorption ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) mass spectrometry (Alphalyse, Odense, Denmark).

NH_2-terminal Edman sequencing

Proteins were separated by SDS-PAGE analysis and electroblotted onto PVDF membranes as described above. After washing with H_2O and staining with Coomassie Brilliant Blue R-250 (Sigma), the protein band(s) of interest were excised from the blot and applied to a capillary protein sequencer (Procise 491 cLC, Applied Biosystems) for NH_2-terminal Edman degradation.

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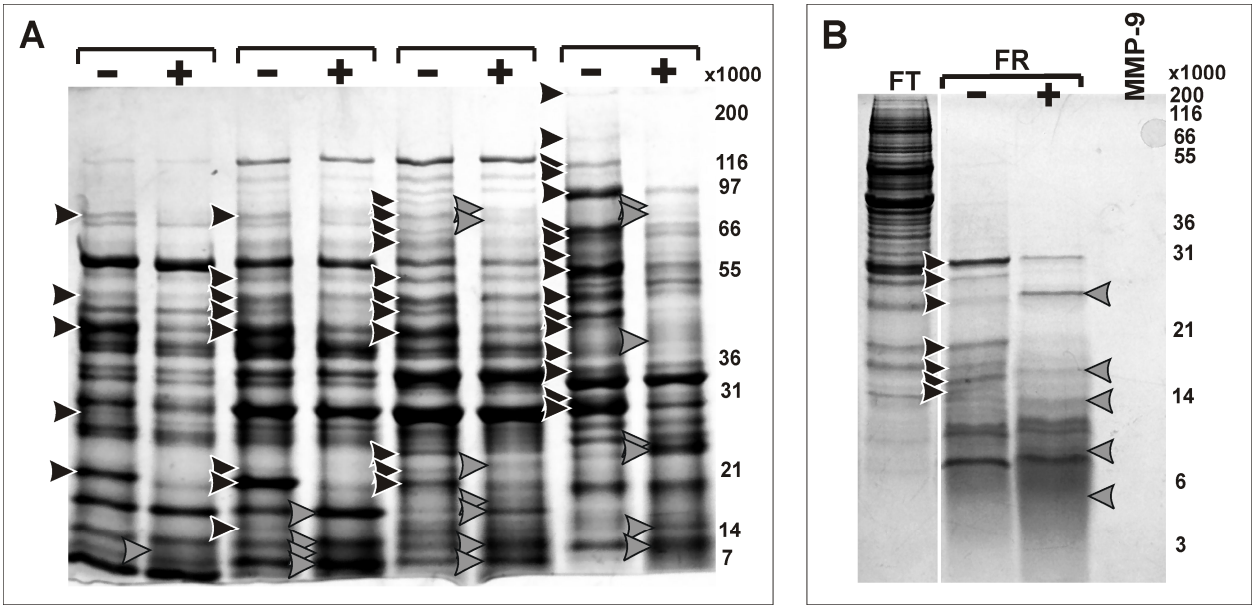

Representative gels generated by 2D-degradomics and fragment degradomics. **A.** After incubation in the absence (-) or presence (+) of MMP-9 (100 nM) for 24h, the ion exchange (IEC) fractions were separated by molecular mass upon SDS-PAGE analysis. Protein bands that were present in the uncleaved fractions (-) but disappeared or decreased in the cleaved fractions (+) were considered to be candidate MMP-9 substrates and indicated by black arrowheads. Protein bands that appeared or increased in intensity in the digested fractions (+) were potential substrate fragments (depicted by grey arrowheads). Apparent molecular masses are shown on the right in Da. **B.** The pooled CEC flow through fractions (FT) were neutralized and incubated with (+) or without (-) activated MMP-9 (100 nM) for 24 h. Fragment fractions (FR) were enriched by fragment degradomics and analyzed by SDS-PAGE. Protein bands that were present in the uncleaved fragment fraction (FR, -) but disappeared or decreased in the cleaved fragment fraction (FR, +) were considered to be candidate MMP-9 substrates (black arrowheads). Protein bands that appeared or increased in intensity in the digested fragment fraction (FR, +) were potential substrate fragments (grey arrowheads). As a control for non-specific MMP-9 fragments, activated MMP-9 (100 nM) was also subjected to fragment degradomics and analyzed on the same gel (lane 4). Apparent molecular masses are shown on the left in Da.

CHAPTER 4. GELATINASE B/MMP-9 SUPPRESSES LPR-INDUCED LYMPHOPROLIFERATION AND LUPUS-LIKE SYSTEMIC AUTOIMMUNE DISEASE.

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GELATINASE B/MMP-9 SUPPRESSES *LPR*-INDUCED LYMPHOPROLIFERATION AND LUPUS-LIKE SYSTEMIC AUTOIMMUNE DISEASE

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ABSTRACT

Gelatinase B/matrix metalloproteinase-9 (MMP-9) is a key enzyme involved in inflammatory, hematological, vascular and neoplastic diseases. Although the absence of MMP-9 has been shown to enhance allograft rejection and T cell alloreactivity, the role of MMP-9 in lymphocyte homeostasis is not well studied. $B6^{lpr/lpr}$ mice with defective Fas-mediated apoptosis were used to investigate the functions of MMP-9 in lymphocyte proliferation and in the development of systemic autoimmunity. Combined Fas and gelatinase B deficiency resulted in extreme lymphoproliferative disease with enhanced lymphadenopathy and splenomegaly, and significantly reduced survival compared with single Fas deficiency. At the cellular level, this was corroborated by increased lymph node accumulation of activated and 'double negative' T cells, B cells and myeloid cells. In addition, higher autoantibody titers and more pronounced autoimmune tissue injury were found in the absence of MMP-9, culminating in chronically enhanced lupus-like autoimmunity. As 'uncleaved' autoantigens seemed to be better stimuli for autoantibody production, MMP-9 may suppress systemic antibody-mediated autoimmunity by clearance of autoepitopes in immunogenic substrates. Thus, new protective functions for MMP-9 were revealed in the suppression of lymphoproliferation and dampening of systemic autoimmunity, cautioning against the long-term use of MMP inhibitors in autoimmune lymphoproliferative syndrome (ALPS) and systemic lupus erythematosus (SLE).

KEYWORDS: protease, lymphoproliferation, apoptosis, systemic autoimmunity, lupus, autoantigen

INTRODUCTION

Gelatinase B or matrix metalloproteinase-9 (MMP-9) is a secreted protease involved in the remodeling of the extracellular matrix and the migration of normal and tumor cells.[1] MMP-9 has physiological roles in reproduction and development, and in the repair of tissues, but is mostly known for its pathological effects in cancer, in vascular diseases and in autoimmune diseases.[2] In contrast with the constitutive presence of gelatinase A (MMP-2), MMP-9 is an inducible protease. In an infectious or inflammatory context, gelatinase B secretion is stimulated by Toll-like receptor (TLR) agonists, cytokines and chemokines and affects immune functions by processing cytokines and chemokines and mobilizing leukocytes.[3]

Organ-specific autoimmune diseases, in which MMP-9 exerts an exacerbating role, are multiple sclerosis[4] - and its animal model of experimental autoimmune encephalomyelitis (EAE)[5]-, bullous pemphigoid[6] and rheumatoid arthritis.[7] Acute exacerbations of these diseases are associated with increased production of MMP-9, whereas knocking out the MMP-9 gene in experimental models diminishes the clinical symptoms of acute relapses.[6,8,9] In terms of antigen processing and lymphocyte activation, organ-specific autoimmune diseases typically are T cell-mediated disorders and the autoantigens are often extracellular proteins.

Little is known, however, about the functional role of MMP-9 in systemic autoimmunity and on long-term effects in chronic autoimmune models. Often, these are B cell- and antibody-mediated diseases and the antigens are intracellular proteins or macromolecules, including double-stranded (ds) and single-stranded (ss) DNA, small nuclear ribonucleic proteins (snRNPs) and heat shock proteins (HSPs).[10,11] In previous studies, we explored the intracellular degradome of MMP-9 and found many intracellular matrix (ICM) proteins and systemic autoantigens as novel intracellular gelatinase B substrates.[12,13] These findings led to the insight that MMPs and other proteases may have novel

(immuno)regulatory properties by the clearance of toxic and immunogenic burdens of (abundant) ICM proteins and intracellular autoantigens released after extensive necrosis (e.g. caused by trauma, burns, frost bite, irradiation). In addition, the prototypic systemic autoimmune disease systemic lupus erythematosus (SLE) is associated with accelerated leukocyte apoptosis[14,15] and impaired clearance of apoptotic cells.[16] Hence, the unphagocytosed apoptotic cell load progresses to secondary necrosis, releasing intracellular proteins and systemic autoantigens into the extracellular milieu.

Besides its roles in leukocyte mobilization and in proteolytic processing of cytokines, chemokines and autoantigens, MMP-9 also functions in adaptive immune reactions. Deficiency of MMP-9 leads to enhanced T cell alloreactivity in heterotypic tracheal and cardiac allografts. This T cell alloreactivity in MMP-9-deficient mice is mediated by enhanced dendritic cell stimulatory as well as enhanced T cell responsive capacities.[17,18] Although expression of MMP-9 by T and B cells is known[2], the direct effects of MMP-9 or its deficiency on activation and proliferation of T and B lymphocytes have not been well studied.

Following the general view that MMP-9 plays functional roles in inducible phenotypes and that MMP-9 influences homeostasis of normal T cells, we investigated the effect of MMP-9 *in vivo* in mice with the *lpr* (lymphoproliferative) loss-of-function mutation in the apoptosis mediator Fas as treshhold-altering event. Fas mutant mice on the MRL background ($MRL^{lpr/lpr}$) develop a severe lupus-like syndrome with vasculitis, arthritis, early-onset splenomegaly, progressive lymphadenopathy and immune complex-mediated glomerulonephritis.[19] However, on the C57Bl/6 background ($B6^{lpr/lpr}$), disease development is subdued and delayed, with moderate lymphoproliferation, later onset of autoantibody production and little immunopathology.[20,21] In the present study, we discovered that the genetic knockout of MMP-9 in $B6^{lpr/lpr}$ mice ($B6^{lpr/lpr}MMP\text{-}9^{-/-}$) resulted in highly augmented and earlier onset lymphoproliferation, increased autoantibody production against multiple autoantigens and more pronounced autoimmune tissue injury. These data suggest that MMP-9 is a protective factor in the development of autoimmune lymphoproliferation.

MATERIALS AND METHODS

Mice

MMP-9 deficient mice ($B6.MMP\text{-}9^{-/-}$) were generated as described previously[8] and backcrossed to the C57Bl/6 strain for ten generations. $B6^{lpr/lpr}$ mice were purchased from Jackson Laboratory (Bar Harbor, Maine, ME, USA). Crossing of $B6.MMP\text{-}9^{-/-}$ and $B6^{lpr/lpr}$ mice yielded F1 heterozygotes that were mated to obtain $B6^{lpr/lpr}MMP\text{-}9^{-/-}$ and $B6^{lpr/lpr}MMP\text{-}9^{+/+}$ in the F2 generation. In each individual mouse, the genotype was confirmed by PCR. Mice were bred in the specific pathogen-free (SPF) breeding facility of the Rega Institute for Medical Research (University of Leuven, Leuven, Belgium) and moved to non-SPF conditions after weaning. All experimental procedures were approved by the institutional Ethics Committee under license LA1210243 for animal welfare (Project 06009).

Genotyping

Genomic DNA was obtained from tail or peripheral blood cells using the EZNA tissue DNA purification kit (Omega Bio-Tek, Inc., Norcross, GA, USA). Detection of MMP-9 deficiency was performed as described previously[8] with slightly modified primers: forward: 5'-TCCATCCACAGGCATAC-TTGTACC-3'; reverse 1 for the knock-out allele: 5'-TGTCAGTTTCATAGCCTGAAGAACG-3'; and reverse 2 for the wild-type allele: 5'-ATCTG-CTGTCCCTTCTACTCTGTC-3'. Denaturation was attained with 5 min at 95°C, followed by 50 amplification cycles (1 min at 95°C, 30 s at 64°C, 1 min at 72°C), and a final extension step of 10 min at 72°C. PCR for the *lpr* mutation was carried out as suggested by Jackson Laboratory. The following nucleotides were used to distinguish the *lpr* allele from the wild-type *Fas* allele:forward 5'-GTAAA-TAATTGTGCTTCGTCAG-3'; reverse 1 for the *lpr* allele: 5'-TAGAAAGGTGCACGGGTGTG-3'; and reverse 2 for the wild-type allele: 5'-CAAATCTAG-GCATTAACA-GTG-3'. PCR conditions were 3 min at 94°C, then 35 cycles (30s at 94°C, 1 min at 59°C, 1 min at 72°C), with a final extension of 7 min at 72°C. The amplified products of the *MMP-9* and *lpr* PCR were separated on a 2% and 3% agarose gel, respectively, and visualized by ethidium bromide staining.

Phenotyping

Peripheral blood samples were incubated with high concentrations (1 μM) of formyl-Methionyl-Leucyl-Phenylalanine (fMLP) (Sigma, St. Louis, MO, USA) for 1h at 37°C to trigger extensive neutrophil degranulation. Plasma samples were obtained by centri-fugation and first prepurified on gelatin-Sepharose beads as described.[22] Gelatin zymography was then performed as previously detailed.[23] The relative migrations of pro-MMP-9/gelatinase B and pro-MMP-2/gelatinase A, and of activated forms, were determined with the use of laboratory standards of deletion mutants of MMP-9.[24]

Macroscopic pathology staging

To summarize pathological observations, a semi-quantitative macroscopic pathology score was established as follows. A score of 0; 0.25; 0.5 or 1 was given in accordance with the degree of swelling of axillary and inguinal lymph nodes (0 to 1); cervical lymph nodes (0 to 1) and mesenteric lymph nodes (0 to 1). A visibly affected liver was scored as 1 (*vs.* 0 for non-affected livers). Enlargement of the thymus was scored with 0.5 or 1.Hence, the total summed macroscopic pathology scores ranged between 0 and 5.

Flow cytometry analysis and antibodies

Single-cell suspensions were prepared from spleen and (axillary and inguinal) lymph nodes using nylon cell strainers (Becton Dickinson, San Jose, CA, USA). Erythrocytes were depleted by hypotonic lysis with NH_4Cl solution (0.083% NH_4Cl in 0.01M Tris/HCl, pH 7.4). Peripheral leukocytes were obtained after two erythrocyte depletions. Cells were washed twice with phosphate buffered saline (PBS) + 2% fetal calf serum (Invitrogen, Carlsbad, CA, USA) and counted. 10^6 cells were first incubated with Fc-receptor blocking antibodies (anti-mouse CD16/CD32; Miltenyi Biotec, Bergisch Gladbach, Germany) followed by washing and staining with combinations of the following anti-mouse antibodies (eBioscience, San Diego, CA, USA): FITC-labeled anti-CD3, anti-GR1, anti-CD11c; PE-labeled anti-B220, anti-F4/80, anti-CD40; APC-labeled anti-CD4 and anti-CD11b, and PerCP-Cy5.5-labeled anti-CD8. Data collection was carried out on a FACSCalibur™ flow cytometer and data were processed with the Cell Quest® software (Becton Dickinson).

Proliferation assays

Single-cell suspensions ($0.5x10^6$ cells/mL), prepared as mentioned before, were incubated in the absence or presence of hamster anti-mouse CD3 (3 μg/mL, purified from 145-2C11 hybridoma cell supernatant[25]) or anti-IgM (5 μg/mL, Jackson ImmunoResearch, West Grove, PA, USA). After 48h of incubation, cells were pulsed for 18h with ^{3}H-thymidine (1 μCi/well, GE Healthcare, Uppsala, Sweden). Finally, cells were harvested and ^{3}H-thymidine incorporation was measured in a liquid scintillation analyzer (Tri-Carb 2100 TR, Packard, Canberra, Australia).

In vitro autoantigen cleavage by MMP-9

Recombinant human MMP-9 was expressed in Sf9 insect cells and purified by gelatin-Sepharose affinity chromatography as described previously.[24] Purified MMP-9 (10 μM) was activated with 0.1 μM of the catalytic domain of human MMP-3 (cd-MMP-3, Calbiochem, Darmstadt, Germany) for 1.5h at 37°C. The autoantigens (100 μg/mL, Diarect, Freiburg, Germany) U1 small nuclear ribonucleoprotein (U1snRNP) 70 kDa, U1snRNP A, Smith antigen (UsnRNP B/B'), ribosomal protein P0 and Ro-52 were incubated for 5h (or 20h) at 37°C in the absence or presence of activated MMP-9 (1.6 to 200 nM), with equivalent amounts of the catalytical domain of MMP-3 (2 nM) used to activate the MMP-9 and with 200 nM activated MMP-9 supplemented with 20 mM of the metalloprotease inhibitor ethylene diaminetetraacetic acid (EDTA). As a control for non-substrate fragments, the highest concentration of activated MMP-9 was incubated separately without addition of substrate. Incubated autoantigen samples were analyzed by SDS-PAGE on 12.5 % acrylamide gels (Prosieve® 50 Gel Solution, Lonza, Basel, Switzerland) and visualized with Coomassie Brilliant Blue R-250 (Sigma).

Measurement of autoantibodies

Autoantibody titers in individual plasma samples were determined by enzyme-linked immunosorbent assay (ELISA) as adapted from Cohen and Maldonado.[26] Briefly, ELISA plates were coated overnight (4°C) with one of the following autoantigens: plasmid DNA (2 μg/mL, Diarect); rabbit IgG (purified on a protein A Sepharose column, GE Healthcare); human Smith antigen (U snRNP B/B', 0.4 μg/mL, Diarect); calf thymus histones (5 μg/mL, Sigma); rabbit skeletal muscle actin (3 μg/mL, Cytoskeleton, Denver, CO, USA); bovine brain tubulin (3 μg/mL, Cytoskeleton) and

chicken erythrocyte chromatin (5 µg/mL) extracted from chicken red blood cells (TCS Biosciences, Buckingham, UK) as described previously.[26] For the U1snRNP ELISA, plates were coated with the three polypeptides that are unique to the U1snRNP complex: U1snRNP 70 kDa, U1snRNP A and U1snRNP C (0.5 µg/mL, Diarect). Plates were blocked with 0.5% bovine serum albumin (BSA, Cohn fraction V, Sigma) + 0.2% Tween-20 (AppliChem, Darmstadt, Germany) in PBS for at least 2h at room temperature and washed. Serial plasma dilutions (in PBS with 0.5% BSA and 0.2% Tween-20) from individual mice were added, incubated overnight at 4°C and washed with PBS containing 0.2% Tween-20. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA) was used as a secondary detection antibody. After 1h of incubation at 37°C, plates were washed and HRP activity was visualized by adding the substrate tetramethylbenzidine. After reaction termination with 2M H_2SO_4, optical densities were determined at 450 nm in a microplate spectrophotometer (Power Wave XS, Biotek, Winooski, VT, USA). Various plasma samples were pooled to form a laboratory standard that was given the value of 100 units. Standard curves were constructed by plotting the laboratory standard dilutions *vs.* the optical densities (ODs), yielding an equation that was used to calculate the autoantibody units in the individual samples.

Competitive ELISA

ELISA was performed as described above but with the following modifications. Plates were coated with intact ribosomal protein P0 or U1snRNP A (0.5 µg/mL). After blocking, dilution series of free autoantigen (previously cleaved by MMP-9 or intact) were added, starting from a 50x excess, followed by addition of the plasma samples. When free antigen (intact or cleaved) competed with coated intact antigen for autoantibody binding, the formed immune complexes were not bound to the plates, resulting in a drop in OD. No addition of free antigen was considered as 0% competition and 100% OD.

Histologic examination of tissues

At 9 months, lymph nodes (axillary, inguinal, cervical, mesenteric, renal, pyloric, lumbal), spleen, thymus, liver, lung and kidneys were excised, weighed and fixed in 10% formalin. Specimens were then inbedded in paraffin, sectioned (4 µm) and stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) stain for the kidney sections. All sections were scored by two pathologists blinded to the study (X.S. and H.H.). Lymph nodes were determined as normal (score 0), with slight (score 1) or moderate (score 2) perturbation of the structure when a visible distinction between cortex and medulla and discernible follicles were still present. A score of 3 was attributed when cortex, medulla and follicles could no longer be discerned. Spleen scores were based on the expansion of the white pulp *vs.* the red pulp, the appearance of nodular structures distorting the white pulp, and lymphocyte infiltration in the red pulp. The overall severity of these combined observations was graded from 0 to 3. Liver and lung scores were determined by the degree of lymphocyte infiltration. A liver infiltration score of 1 represented a moderate periportal lymphocytic infiltration, whereas a score of 2 was given in case of a severe periportal and parenchymal infiltration. For lung infiltration, score 1 was attributed to a moderate peribronchial infiltration; score 2 to a considerable peribronchial and alveolar lymphocyte infiltration and an (additive) score of 3 for bronchiolitis obliterans organizing pneumonia (BOOP). For immunohistochemistry, slides were stained with anti-CD3, anti-B220, anti-CD4, anti-CD8, anti-IgM, anti-IgD and anti-CD68 (Dako, Glostrup, Denmark) as previously described.[27]

As initially no kidney samples were fixed for electron microscopy, small samples from the frozen blocs were thawed and fixed in 2.5% glutaraldehyde, 0.1 M phosphate buffer, pH 7.2 at 4°C overnight. After 1h post-fixation in 2% osmium tetroxide, 0.1 M phosphate buffer, pH 7.2 at 4° C, the samples were dehydrated in graded series of alcohol and embedded in epoxy resin. Ultra-thin sections of 50 to 60 nm were cut, stained with uranyl acetate and lead citrate and examined at 50 kV using a Zeiss EM 900 electron microscope (Oberkochen, Germany). Images were recorded digitally with a Jenoptik Progress C14 camera system (Jena, Germany) operated using Image-Pro express software (Media Cybernetics, USA).

Assessment of renal function

Microalbuminuria was determined by sandwich ELISA using a standard curve of known concentrations of mouse serum albumin (MSA, Sigma). Briefly, ELISA plates were coated overnight (4°C) with goat anti-mouse albumin (United States Biological, Swampscott, MA, USA), blocked with PBS containing 0.5% and 0.2% Tween-20 and washed (with PBS + 0.1% Tween-20). Dilutions of urine samples and a standard of MSA were applied, incubated overnight at 4°C and washed. Detection with goat anti-mouse albumin (United States Biological) as a secondary antibody and calculations were carried out as detailed in 'Measurement of autoantibodies'. Urinary

creatinine levels were measured with the Quantichrom™ Creatinine Assay Kit (BioAssay Systems, Hayward, CA, USA) according to the manufacturers recommendations. Albumin-to-creatine ratios (ACR) were obtained by normalizing the albumin concentrations to the creatinine levels.

Patients samples

All patients contributing to the present study gave informed consent and the study protocol was in accordance with international standards and approved by the Local Ethical Committee for clinical studies.

Statistical analysis

Data were analyzed with GraphPad Prism 5.02 (GraphPad, San Diego, CA, USA). Graphs combine a scatter dot plot of individual data points with bars or lines representing the medians. Differences between two groups were analyzed by the nonparametric Mann-Whitney U-test. Proportions were compared with the Fisher's exact test. Survival and cumulative incidence were analysed by the Log-Rank (Mantel Cox) Test. Associations were determined by two-tailed Spearman rank correlation. Two-tailed *P* values less than .05 were considered statistically significant.

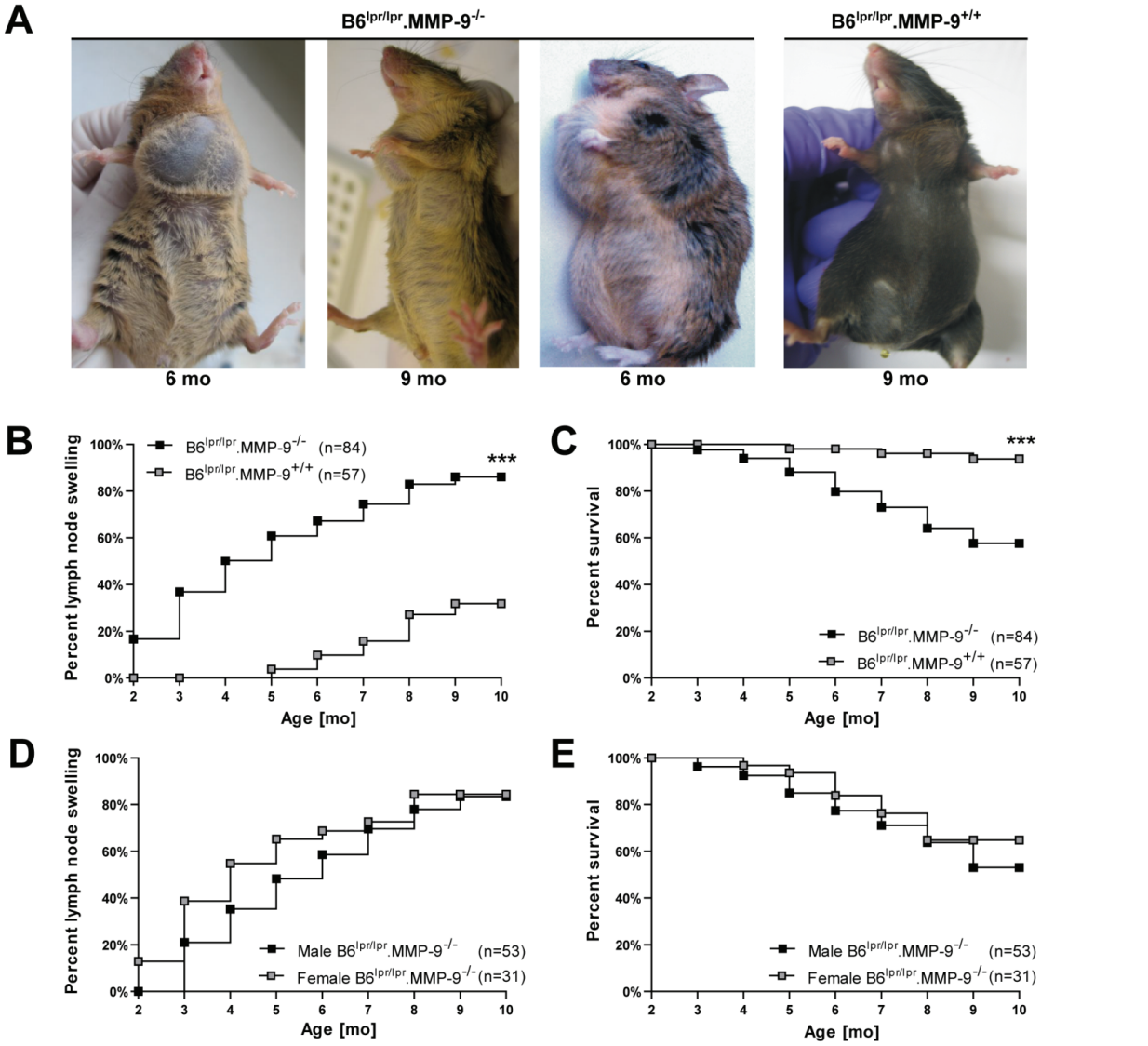

Figure 1. Enhanced lymph node swelling and decreased survival in B6$^{lpr/lpr}$.MMP-9$^{-/-}$ mice *vs.* B6$^{lpr/lpr}$.MMP-9$^{+/+}$ mice. (A) Representative pictures of pronounced lymphadenopathy at the chest and axills in MMP-9-deficient and MMP-9-sufficient B6$^{lpr/lpr}$ mice. The age of each individual mouse is shown at the bottom of the picture. Cumulative incidence of lymph node swelling was compared over an observation period of 10 months (B) between B6$^{lpr/lpr}$.MMP-9$^{-/-}$ mice and B6$^{lpr/lpr}$.MMP-9$^{+/+}$ mice and (D) between male and female B6$^{lpr/lpr}$.MMP-9$^{-/-}$ mice. Survival was monitored until the age of 10 months in (C) B6$^{lpr/lpr}$.MMP-9$^{-/-}$ mice *vs.* B6$^{lpr/lpr}$.MMP-9$^{+/+}$ mice and (E) in male *vs.* female B6$^{lpr/lpr}$.MMP-9$^{-/-}$ mice. The numbers of mice (n) in each group are depicted in the graph legend. ***, $P < .0001$, cumulative readout by the Log-Rank (Mantel Cox) Test.

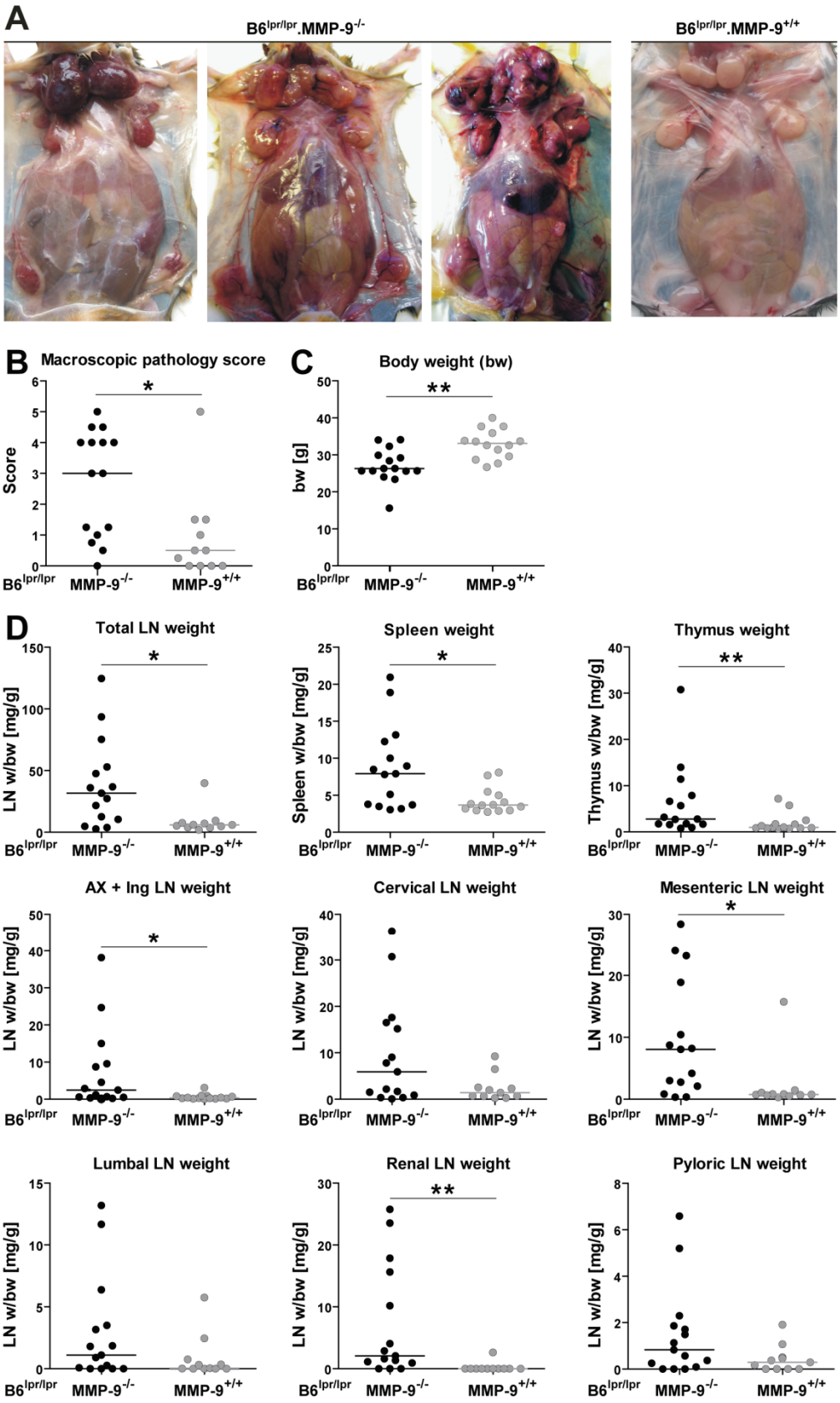

Figure 2. Enhanced lymphadenopathy and macroscopic pathology in B6$^{lpr/lpr}$.MMP-9$^{-/-}$ mice *vs.* control mice. (A) Dissection analysis of MMP-9-deficient and MMP-9-sufficient B6$^{lpr/lpr}$ mice with pronounced lymphadenopathy, revealing enlargement of cervical, axillary and inguinal lymph nodes. (B) Pathological findings of the dissection analysis were summarized in a macroscopic pathology score. (C) Body weight (bw) as well as (D) spleen, thymus and various lymph node (LN) weights normalized over the respective body weights (w/bw, weight per body weight) were compared between B6$^{lpr/lpr}$.MMP-9$^{-/-}$ mice and B6$^{lpr/lpr}$.MMP-9$^{+/+}$ mice. AX + Ing LN, axillary and inguinal lymph nodes. Horizontal lines indicate the group medians. Each dot represents data from a single animal. *$P < .05$, **$P < .01$.

RESULTS

Lack of MMP-9 induces severe lymphoproliferation and decreased survival in B6$^{lpr/lpr}$ mice

With the aim of investigating the influence of MMP-9 in the development of lymphoproliferation and systemic autoimmunity in B6$^{lpr/lpr}$ mice, we generated mice lacking both MMP-9 and functional apoptosis-inducing receptor Fas (B6$^{lpr/lpr}$.MMP-9$^{-/-}$) (with both parental strains backcrossed onto a C57Bl/6 background for 10 generations). Zymographic analysis of plasma samples after induction of neutrophil degranulation confirmed the phenotype of MMP-9 deficiency (Supplementary Figure 1). While the B6$^{lpr/lpr}$ mice developed moderate lymph node swelling as expected, many B6$^{lpr/lpr}$.MMP-9$^{-/-}$ mice developed extreme lymphadenopathy at the chest and axills (Figure 1A). Onset of lymph node swelling was advanced and the overall cumulative incidence was markedly increased in the absence of MMP-9 ($P < .0001$) (Figure 1B). In addition, survival was significantly reduced in a large-size B6$^{lpr/lpr}$.MMP-9$^{-/-}$ cohort over an observation period of 10 months ($P < .0001$) (Figure 1C). No differences in lymph node swelling or mortality were observed between male and female B6$^{lpr/lpr}$.MMP-9$^{-/-}$ mice (Figure 1D-E).

Dissection of 9 months old mice showed that the externally observed lymphadenopathy was caused by extreme enlargement of cervical, axillary and inguinal lymph nodes, while B6$^{lpr/lpr}$ mice showed moderate or hardly visible swelling (Figure 2A). Besides the superficial lymph nodes, renal, mesenteric, lumbar and pyloric lymph nodes were also markedly enlarged and often highly vascularized or even hemorrhagic in B6$^{lpr/lpr}$.MMP-9$^{-/-}$ mice (Supplementary Figure 2A-C). Extreme lymphadenopathy was frequently associated with splenomegaly, enlargement of the thymus, and a visibly affected liver with blunt edges and a granular surface (Supplementary Figure 2D, E and F, respectively). To summarize these pathological findings in a macroscopic pathology score, we developed a semi-quantitative staging system, as outlined in 'Materials and methods'. The results showed significantly increased pathological stages ($P < .05$) in the absence of MMP-9 (Figure 2B).

In order to corroborate the observed amplification in lymphoproliferation with quantitative data, organ and lymph node weights from both groups of mice were compared. Previously, it has been demonstrated that MMP-9$^{-/-}$ mice have a spontaneous phenotype of impaired bone formation and reduced body weight.[28] As in the present study the total body weights of B6$^{lpr/lpr}$.MMP-9$^{-/-}$ mice were also decreased *vs.* B6$^{lpr/lpr}$.MMP-9$^{+/+}$ mice ($P < .01$) (Figure 2C), we normalized the organ weights over the respective body weights. Higher lymph node ($P < .05$), spleen ($P < .05$) and thymus weights ($P < .01$) confirmed the severe lymphoproliferation caused by MMP-9 deficiency (Figure 2D). In the analysis of lymph nodes at different anatomical sites, this trend persisted and was significant for axillary and inguinal, for mesenteric, and for renal lymph nodes. Of note, lung, liver and kidney weights were not significantly different between B6$^{lpr/lpr}$.MMP-9$^{-/-}$ and B6$^{lpr/lpr}$.MMP-9$^{+/+}$ mice.

The accumulation of T and B cells in peripheral lymphoid organs is more pronounced in the absence of MMP-9

The peripheral repertoire of mice bearing Fas with the *lpr* mutation is predominated by unusual CD3$^+$B220$^+$CD4$^-$CD8$^-$ double negative (DN) T cells.[21] A recent study showed that very low percentages of these DN T cells exist in the periphery of normal wildtype mice (1% to 5%) and these cells die at very high rates by Fas-mediated apoptosis. In the absence of functional Fas, they progressively accumulate in lymph nodes and spleen causing lymphadenopathy and splenomegaly.[29] The increased lymphadenopathy observed in B6$^{lpr/lpr}$.MMP-9$^{-/-}$ *vs.* B6$^{lpr/lpr}$.MMP-9$^{+/+}$ mice was further confirmed by increased cell numbers in lymph nodes and spleens ($P < .01$), consisting mainly of DN T cells (Figure 3 and Supplementary Figure 3). In the absence of MMP-9, macroscopic lymphadenopathy was more pronounced than splenomegaly, which was reflected by massive DN T cell accumulation and higher numbers of CD4$^+$ and CD8$^+$ T cells and B cells (B220$^+$CD3$^-$) in the lymph nodes. In the peripheral blood, cell numbers were rather constant. DN T cells only showed an increasing trend in B6$^{lpr/lpr}$.MMP-9$^{-/-}$ mice (Figure 3). Although DN T cells are anergic *in vitro*[21] and do no proliferate *in vivo* in lymph nodes and spleen[29], the massive expansion of DN T cells suggested that MMP-9 deficiency might induce proliferation (as observed with alloreactive T cells[17,18]). However, polyclonal stimulation of T and B cells derived from lymph nodes and spleen showed very low *in vitro* proliferation of B6$^{lpr/lpr}$ cells in comparison with T and B cells from B6.MMP-9$^{-/-}$ and B6 mice (Supplementary Figure 4), especially for the T cells, confirming DN T cell anergy. No significant differences in proliferation were observed with or without MMP-9 in both B6$^{lpr/lpr}$ and B6 control mice.

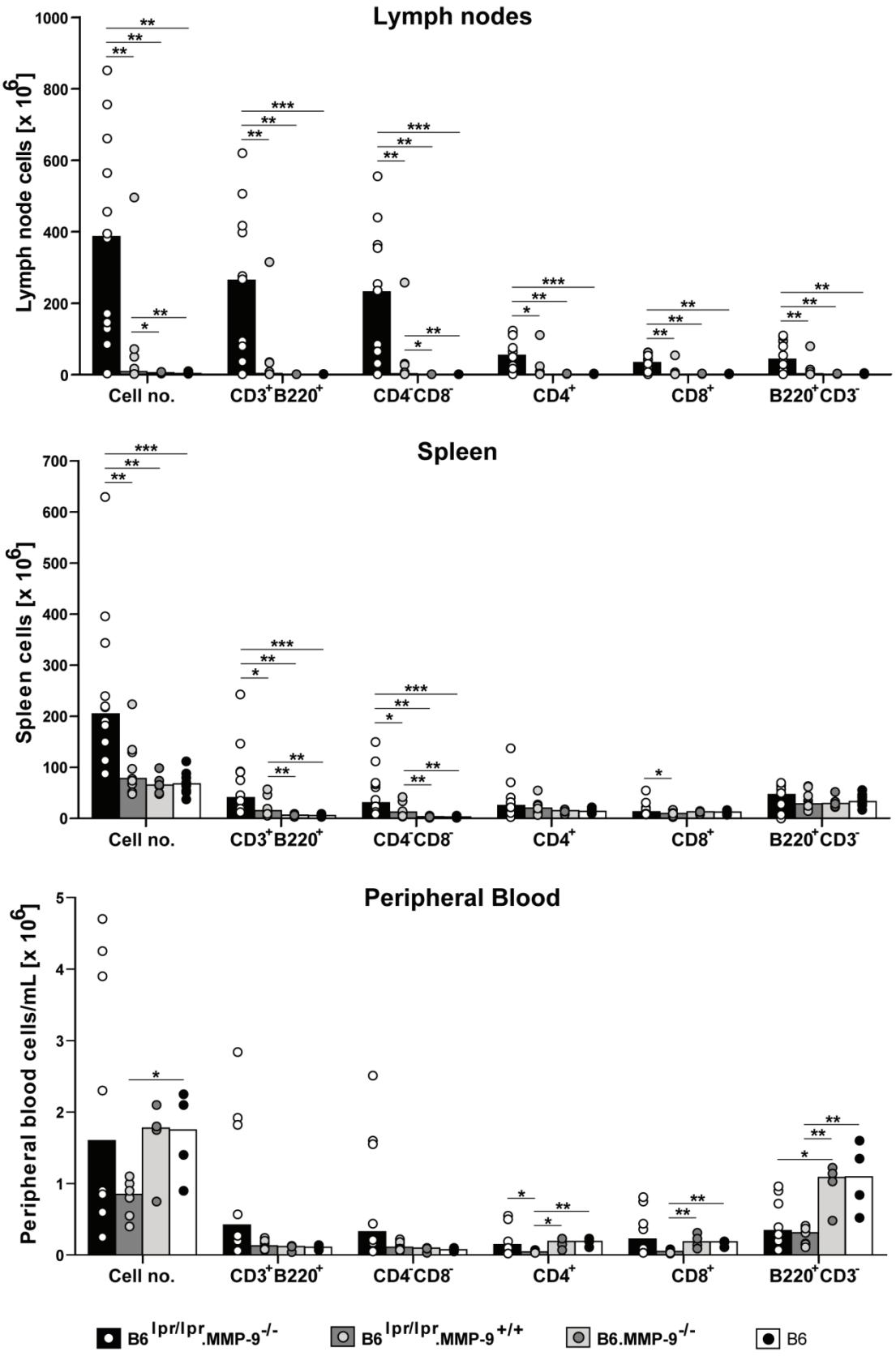

Figure 3. Peripheral lymphoid organs of B6$^{lpr/lpr}$.MMP-9$^{-/-}$ mice show increased infiltration by double negative (DN) T cells in comparison with control mice. Axillary and inguinal lymph node cells (top), splenocytes (middle) and peripheral blood cells (bottom) were isolated from 6 to 9 months old mice (age-matched groups) and stained as detailed in 'Flow cytometry and antibodies'. Double negative (DN) T cells were obtained by gating on CD3$^+$B220$^+$ cells followed by gating on CD4$^-$CD8$^-$ cells. Histograms show from left to right: total cell numbers (Cell no.), total numbers of CD3$^+$B220$^+$ cells, DN T cells (CD4$^-$CD8$^-$), CD4$^+$ T cells, CD8$^+$ T cells and B220$^+$CD3$^-$ B cells. Data from B6.MMP-9$^{-/-}$ and B6 mice are also included for comparison. Histograms indicate group medians and aligned scatter plots show the range of the individual data points. *$P < .05$, **$P < .01$, ***$P < .001$.

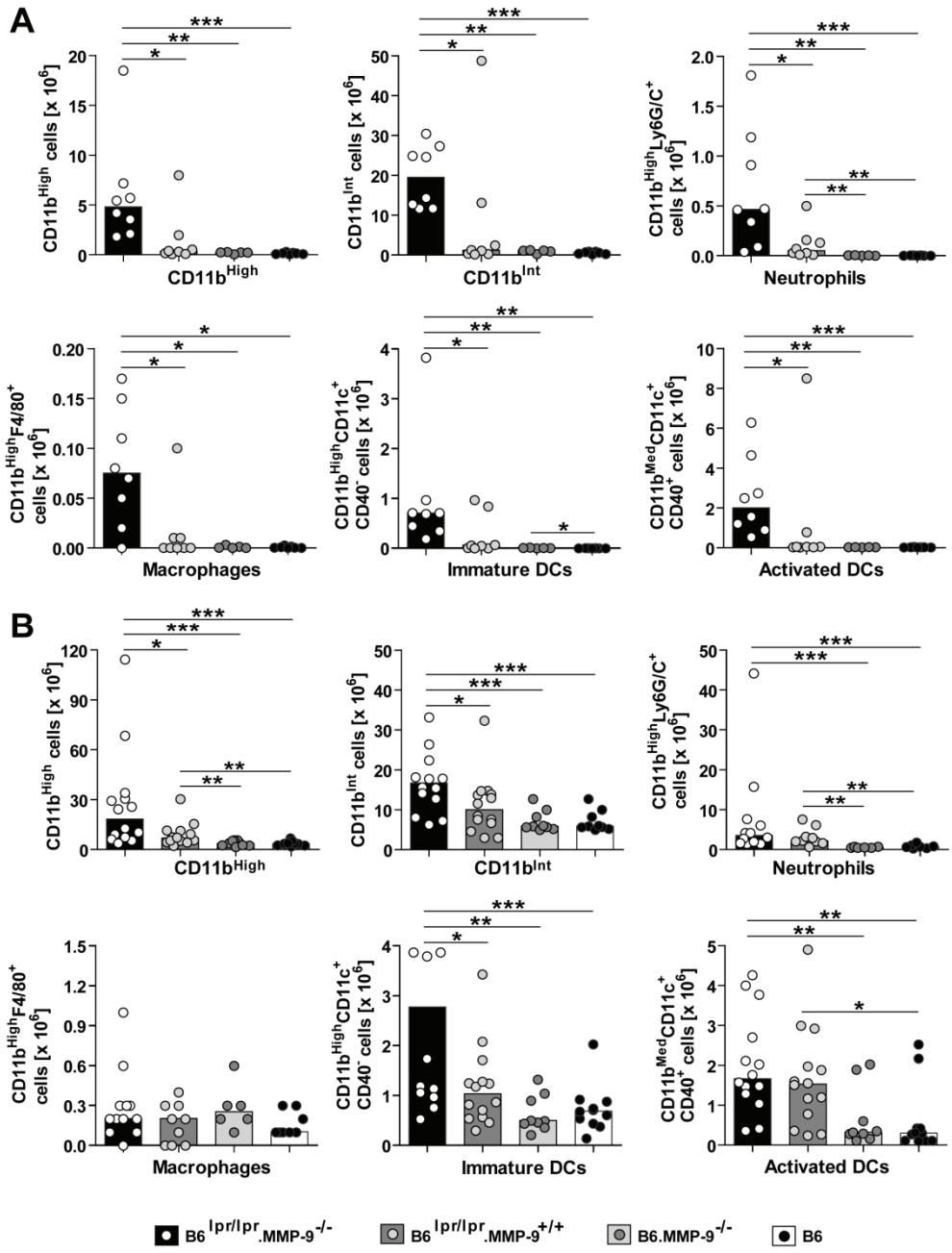

Figure 4. Peripheral lymphoid organs of B6$^{lpr/lpr}$.MMP-9$^{-/-}$ mice *vs.* control mice show increased infiltration by myeloid cell subsets. (A) Axillary and inguinal lymph node cells and (B) splenocytes were isolated from 6 to 9 months old mice (age-matched groups) and stained as described in 'Flow cytometry and antibodies'. Total numbers of neutrophils (CD11b^{High}Ly6G/C^{+}), macrophages (CD11b^{High}F4/80^{+}), immature dendritic cells (DCs, CD11b^{High}CD11c^{+}CD40^{-}) and activated dendritic cells (CD11b^{High}CD11c^{+}CD40^{+}) are depicted in the graphs and compared between groups. Histograms indicate group medians and each dot represents data from a single animal. *$P < .05$, **$P < .01$, ***$P < .001$.

MMP-9 deficiency increases myeloid cell numbers in lymph nodes and spleen

Myeloid cells, such as neutrophils, macrophages and dendritic cells are the major producers of MMP-9 during inflammation.[3] Hence, we wondered whether the absence or presence of MMP-9 would affect myeloid cell subsets in the periphery of B6$^{lpr/lpr}$ mice. Populations with high and intermediate expression of CD11b were significantly increased in the periphery of B6$^{lpr/lpr}$.MMP-9$^{-/-}$ *vs.* the three groups of genetically appropriate control mice (Figure 4). This expansion was again more pronounced in the lymph nodes (Figure 4A) of B6$^{lpr/lpr}$.MMP-9$^{-/-}$ mice compared with the spleens of these mice (Figure 4B). Further analysis showed significantly increased neutrophil, macrophage, immature and activated dendritic cell numbers in the lymph nodes of B6$^{lpr/lpr}$.MMP-9$^{-/-}$ mice, whereas in the spleen only the immature dendritic cells were increased in comparison with B6$^{lpr/lpr}$ and control mice.

Table 1. Correlation between autoantibody titers at 9 months and severity of lymphoproliferation or macroscopic pathology.

Autoantibody specificity	No.†	LN w/bw	Spleen w/bw	MPS
B6$^{lpr/lpr}$.MMP-9$^{-/-}$				
anti-dsDNA IgG	13	0,65 *	0,64 *	0,69 **
anti-chromatin IgG	11	0,28	0,35	0,59
Rheumatoid factor Ig	13	0,54	0,32	0,47
anti-Histone IgG	13	0,70 **	0,63 *	0,63 *
anti-U1snRNP IgG	13	0,61 *	0,59 *	0,73 **
anti-Smith IgG	13	0,68 *	0,60 *	0,60 *
anti-actin IgG	13	0,79 **	0,69 **	0,75 **
anti-tubulin IgG	13	0,74 **	0,65 *	0,84 ***
B6$^{lpr/lpr}$.MMP-9$^{+/+}$				
anti-dsDNA IgG	10	0,50	0,09	0,73 *
anti-chromatin IgG	11	0,37	0,64 *	0,22
Rheumatoid factor Ig	11	0,22	0,36	-0,11
anti-Histone IgG	11	0,58	0,23	0,18
anti-U1snRNP IgG	10	0,24	-0,10	0,64 *
anti-Smith IgG	10	0,48	0,31	0,52
anti-actin IgG	10	0,32	0,09	0,22
anti-tubulin IgG	10	0,32	-0,12	0,29

Depicted are Spearman correlation coefficients with their significance levels, which are indicated by *$P < .05$, **$P < .01$, ***$P < .001$.
†Number of pairs analysed by the Spearman rank correlation test.
LN, lymph nodes; MPS, macroscopic pathology score; w/bw, weight/body weight.

MMP-9 suppresses the production of autoantibodies in B6$^{lpr/lpr}$ mice

Since MMP-9 was shown to cleave many systemic autoantigens[12,13], MMP-9 function may affect development of systemic autoimmunity. Hence, autoantibody titers in plasma samples of B6$^{lpr/lpr}$ mice and control mice were analysed by specific ELISAs and compared. Titers of rheumatoid factor and autoantibodies against dsDNA, chromatin and nuclear autoantigens showed a clear age-dependent increase in B6$^{lpr/lpr}$.MMP-9$^{-/-}$ mice and were significantly higher than those of B6$^{lpr/lpr}$.MMP-9$^{+/+}$ and control mice (B6.MMP-9$^{-/-}$ and B6), the latter showing no significant autoantibody production, as expected (Figure 5). Interestingly, most autoantibody titers in 9 months old B6$^{lpr/lpr}$.MMP-9$^{-/-}$ mice (except for anti-chromatin IgG and rheumatoid factor IgG) showed positive and significant correlations with the degree of lymphoproliferation (as defined by lymph node and spleen weights) and with the macroscopic pathology score (Table 1). In contrast, in B6$^{lpr/lpr}$.MMP-9$^{+/+}$ mice, only a few significant correlations were found, suggesting that the absence of MMP-9 links lymphoproliferation to autoantibody production. Correlations between autoantibody titers and the weights of other organs were not detected.

Since our previous study showed cleavage of SLE autoantigens by MMP-9, various autoantigens used for the ELISAs were incubated in the absence or presence of activated MMP-9. Interestingly, dose-dependent cleavages of U1snRNP A and Smith antigen (UsnRNP B/B') by MMP-9 were observed after 5h of incubation (Figure 6A), whereas U1snRNP 70 kDa was hardly cleaved, even after a longer duration of incubation (20h, data not shown). In addition, other major SLE autoantigens such as ribosomal protein P0 and Ro-52 (Sjögren's syndrome antigen A or SSA) were also efficiently cleaved by MMP-9 after 5h of incubation (Figure 6B). As 'uncleaved' autoantigens seemed to result in higher autoreactive B cell stimulation in B6$^{lpr/lpr}$.MMP-9$^{-/-}$ mice, autoantibodies against two previously identified MMP-9 substrates, actin and tubulin[13], were also evaluated in all groups of mice. Autoantibody levels against actin and tubulin were significantly higher in B6$^{lpr/lpr}$ mice lacking MMP-9 (Figure 6C).

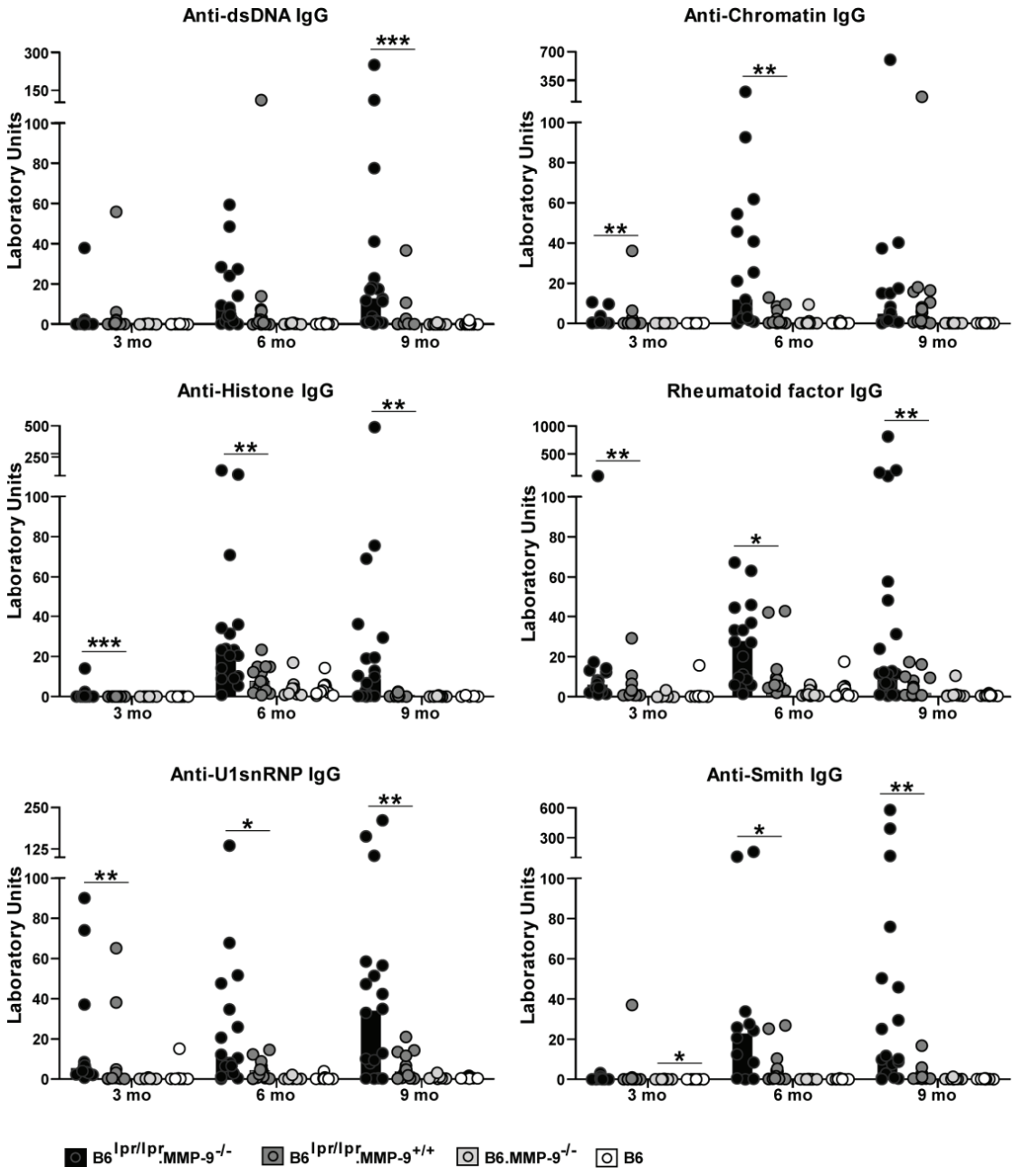

Figure 5. Autoantibody titers are significantly higher in B6$^{lpr/lpr}$.MMP-9$^{-/-}$ mice *vs.* control mice. Plasma samples from four groups of genetically different mice were taken at 3 and 6 or 9 months. Autoantibody titers were determined by ELISA as detailed in 'Measurement of autoantibodies' and compared to a laboratory standard of 100 units. Horizontal lines indicate group medians and each dot represents the titration of serum level from a single animal. Indications of statistical significance are only shown for the comparison between B6$^{lpr/lpr}$.MMP-9$^{-/-}$ and B6$^{lpr/lpr}$.MMP-9$^{+/+}$ mice, since both these groups always showed significantly higher titers in comparison with B6.MMP-9$^{-/-}$ and B6 mice, with exception of anti-dsDNA IgG at 6 months and anti-histone IgG at 3 months, which were not significantly different between B6$^{lpr/lpr}$.MMP-9$^{+/+}$ compared with B6.MMP-9$^{-/-}$ and B6 mice, respectively. No significant differences in autoantibody titers were observed between B6.MMP-9$^{-/-}$ and B6 mice, except for anti-Smith IgG titers, which were significantly higher in 3 months old B6.MMP-9$^{-/-}$ *vs.* B6 mice. *$P < .05$, **$P < .01$, ***$P < .001$.

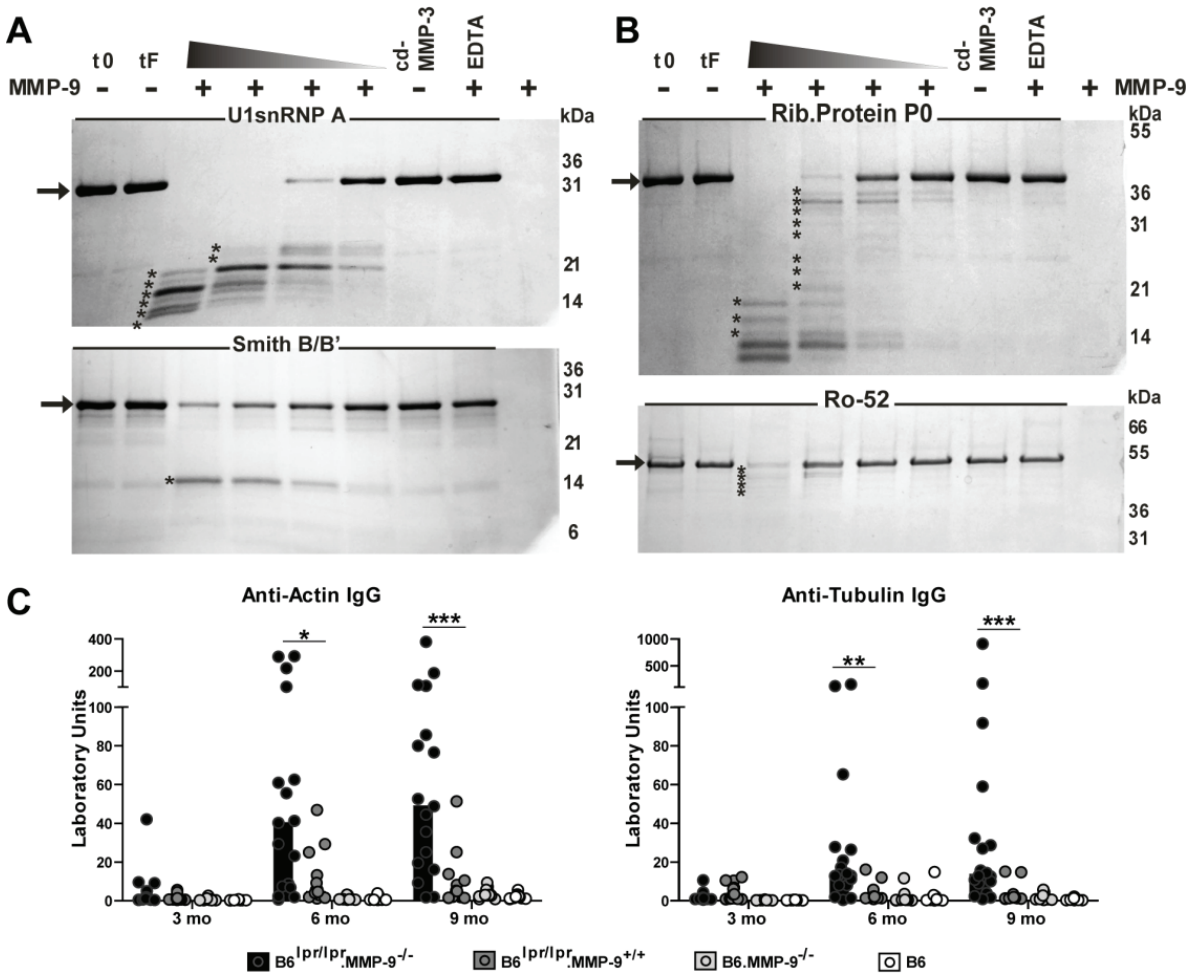

Figure 6. Autoantibody responses against MMP-9 substrates are higher in B6$^{lpr/lpr}$ mice lacking MMP-9. The autoantigens (A) U1snRNP A and Smith antigen (UsnRNP B/B'), as well as (B) ribosomal protein P0 and Ro-52 were incubated for 5h in the absence (tF, -) or presence (+) of various concentrations of activated MMP-9 (200 nM to 1.6 nM, decreasing from lane 3 to 6) and analysed by SDS-PAGE. Control incubations included incubation with activated MMP-9 (200 nM) supplemented with the metalloprotease inhibitor EDTA (20 mM) and incubation with the catalytical domain of MMP-3 (cd-MMP-3, 2 nM), used to activate MMP-9. In addition, the highest concentration of MMP-9 was also incubated separately as a control for non-substrate fragment bands (lane 9). Visualization of the intact substrate (arrow) before incubation is shown under t0. Fragments generated by MMP-9 are indicated with an asterisk (*). Apparent molecular masses are shown next to the gels in kDa. (C) Anti-actin and anti-tubulin IgG titers in plasma samples from 3, 6 and 9 months old mice from the four different genotype cohorts were determined by ELISA as detailed in 'Measurement of autoantibodies' and compared to a laboratory standard of 100 units. Histograms indicate group medians and each dot represents data from a single animal. Indications of statistical significance are only shown for the comparison between B6$^{lpr/lpr}$.MMP-9$^{-/-}$ and B6$^{lpr/lpr}$.MMP-9$^{+/+}$ mice, since both these groups always showed significantly higher titers in comparison with B6.MMP-9$^{-/-}$ and B6 mice, except for anti-actin IgG at 9 months and anti-tubulin IgG at 6 and 9 months, which were not significantly different between B6$^{lpr/lpr}$.MMP-9$^{+/+}$ compared with B6.MMP-9$^{-/-}$ or B6 mice. No significant differences in autoantibody titers were observed between B6.MMP-9$^{-/-}$ and B6 mice. *$P < .05$, **$P < .01$, ***$P < .001$.

To analyze whether MMP-9-cleaved autoantigens are less well recognized by autoantibodies compared with intact antigens, we used competitive ELISA (see 'Materials and methods') since the direct coating of cleaved material is impaired. In competitive ELISA an excess of free autoantigen, either intact or cleaved, competed with coated intact antigen for antibody recognition. In this way, it became clear that after cleavage by MMP-9 ribosomal protein P0 and U1snRNP A are hardly recognized by plasma samples of both B6$^{lpr/lpr}$.MMP-9$^{-/-}$ and B6$^{lpr/lpr}$.MMP-9$^{+/+}$ mice (Figure 7A), pointing to a destruction of B cell epitopes by MMP-9-mediated proteolysis. In addition, the same loss of immunodominant epitopes was observed with autoantibody samples from SLE patients (Figure 7B). Hence, these data suggest that intact substrates of MMP-9 are better triggers for autoantibody production and that MMP-9 exerts a protective effect against autoantibody formation by degradation of these antigenic stimuli.

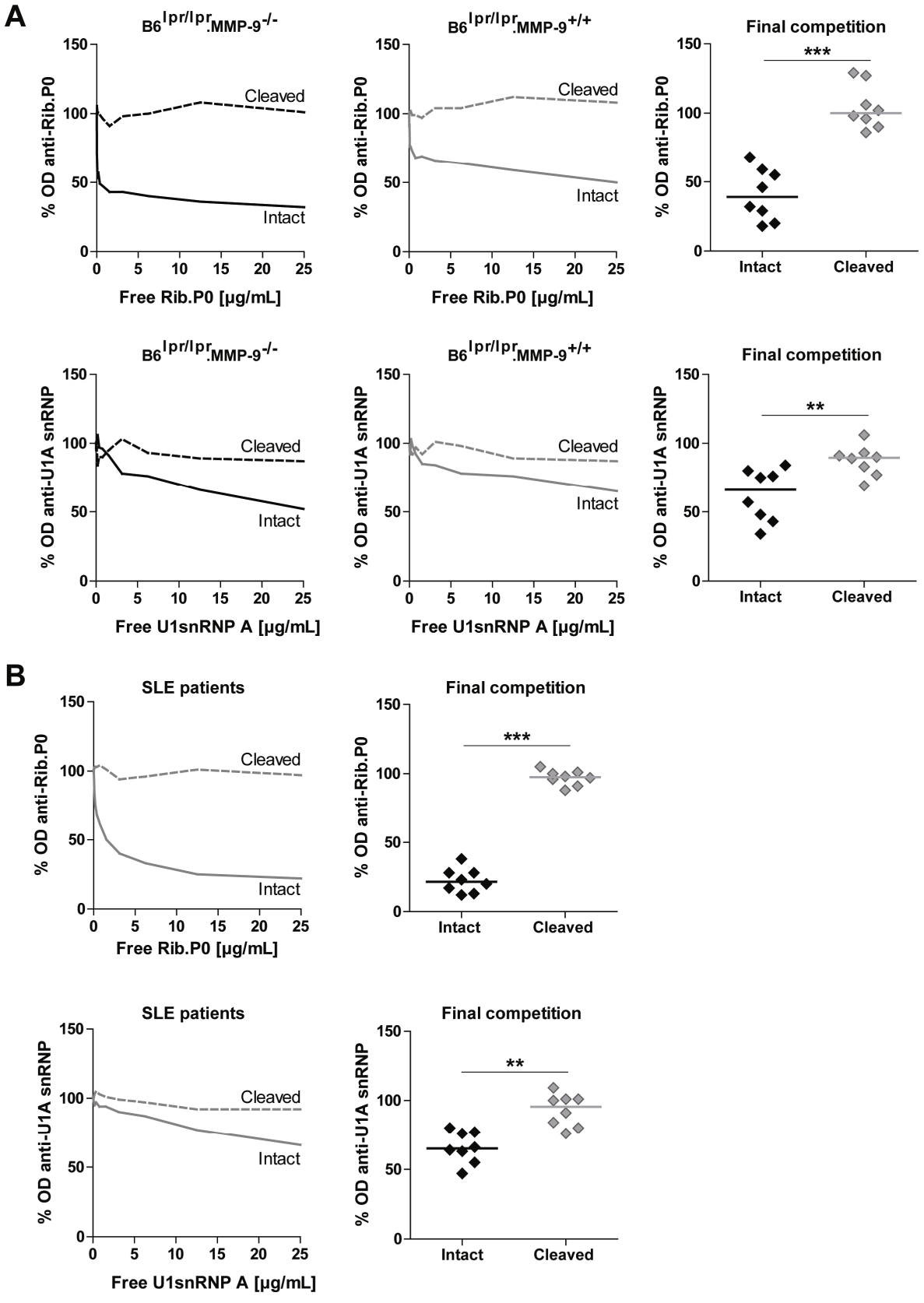

Figure 7. Loss of B cell epitopes in ribosomal protein P0 and U1snRNP A after proteolysis by MMP-9. Binding of autoantibodies from (A) $B6^{lpr/lpr}$ mice and (B) SLE patients to intact (conformational) antigens and MMP-9-generated proteolytic fragments was measured by competitive ELISA. Plates were coated with intact ribosomal protein P0 or U1snRNP A. Increasing concentrations of free autoantigen (previously cleaved by MMP-9 or intact) competed with coated intact autoantigen for autoantibody binding. Immune complexes formed with free autoantigen were not retained on the plates, resulting in a drop in OD. No addition of free antigen (0 µg/mL) was considered as 0% competition and 100% OD. Final competition was considered as the loss of OD (% OD) after addition of the maximal concentration of free autoantigen (25 µg/mL, ratio of free/coated antigen equals 50/1). $^{**}P < .01$, $^{***}P < .001$.

B6$^{lpr/lpr}$MMP-9$^{-/-}$ mice show more pronounced autoimmune tissue injury

To assess the effects of lymphoproliferation and systemic autoimmunity on autoimmune tissue injury, histological examination of organs of 9 months old mice was undertaken. Both in the lymph nodes and in the spleen a complete distortion of the lymphoid architecture was visible in various B6$^{lpr/lpr}$.MMP-9$^{-/-}$ mice but not in B6$^{lpr/lpr}$.MMP-9$^{+/+}$ and other control mice (Figure 8A). In the lymph nodes the distinction between cortex and medulla was blurred and follicles could no longer be discerned. In the spleens, the white pulp architecture was completely disturbed by the massive accumulation of DN T cells forming irregular nodules (CD3^{+}B220^{+}CD4^{-}CD8^{-}IgM^{-}IgD^{-}CD68^{-}, as determined by immunohistochemistry, data not shown) and the red pulp showed massive lymphocyte infiltration. General lymph node and spleen pathology scores (determined as described in 'Materials and methods') were increased in the absence of MMP-9 (Figure 8C), albeit not significantly, except for the renal lymph node score ($P < .05$) (data not shown).

Histological examination of liver, lungs and kidneys revealed increased lymphocyte infiltrations in B6$^{lpr/lpr}$.MMP-9$^{-/-}$ mice in comparison with B6$^{lpr/lpr}$.MMP-9$^{+/+}$ mice. A higher proportion of B6$^{lpr/lpr}$.MMP-9$^{-/-}$ mice showed a moderate or severe periportal and/or parenchymal liver infiltration (40% *vs*. 9%, respectively), and moderate or high peribronchial and/or alveolar lymphocyte infiltration (80% *vs*. 56%, respectively). In various mice these chronic mononuclear cell infiltrations culminated in bronchiolitis obliterans organizing pneumonia (BOOP).

The kidneys of the most affected B6$^{lpr/lpr}$.MMP-9$^{-/-}$ mice showed signs of mesangial proliferative lupus nephritis, whereas no mesangial proliferation was observed on kidney sections of B6$^{lpr/lpr}$.MMP-9$^{+/+}$ mice (Figure 8A). In some of the most affected B6$^{lpr/lpr}$.MMP-9$^{-/-}$ kidneys, immune complex deposits were found by electron microcopy (Figure 8B). In addition, the albumin-to-creatinine ratio (ACR) was significantly higher in the absence of MMP-9 ($P < .05$) (Figure 8C), pointing to glomerular barrier failure in B6$^{lpr/lpr}$.MMP-9$^{-/-}$ mice. A higher incidence of thymuses with cortical thymocyte loss was found in B6$^{lpr/lpr}$.MMP-9$^{-/-}$ mice *vs*. B6$^{lpr/lpr}$.MMP-9$^{+/+}$ mice (44% *vs*. 10%, respectively) (data not shown).

In line with previous data, i.e. absence of histological differences between B6.MMP-9$^{-/-}$ and B6.MMP-9$^{+/+}$ mice[8], we did not observe any differences at 9 months (data not shown). Microscopic and macroscopic pathology scores showed positive associations in B6$^{lpr/lpr}$.MMP-9$^{-/-}$ mice (Supplementary Table 1). Likewise, the extent of lymphoproliferation was significantly correlated with lymph node, spleen and liver pathology scores (and positively but non-significantly with lung pathology scores). However, in B6$^{lpr/lpr}$.MMP-9$^{+/+}$ mice the macroscopic pathology score and degree of lymphoproliferation were only positively (non-significantly) correlated with liver and lung scores. These data suggest that lymphadenopathy and splenomegaly caused more widespread organ injury in the absence than in the presence of MMP-9. As expected form the positive correlations between lymphoproliferation and autoantibody titers (Table 1), histopathology scores (except for the lung pathology scores) also showed positive correlations with autoantibody titers (except for anti-chromatin IgG) in B6$^{lpr/lpr}$.MMP-9$^{-/-}$ mice, but not in B6$^{lpr/lpr}$.MMP-9$^{+/+}$ mice (Supplementary Table 2), suggesting that the absence of MMP-9 results in autoantibody-mediated tissue injury.

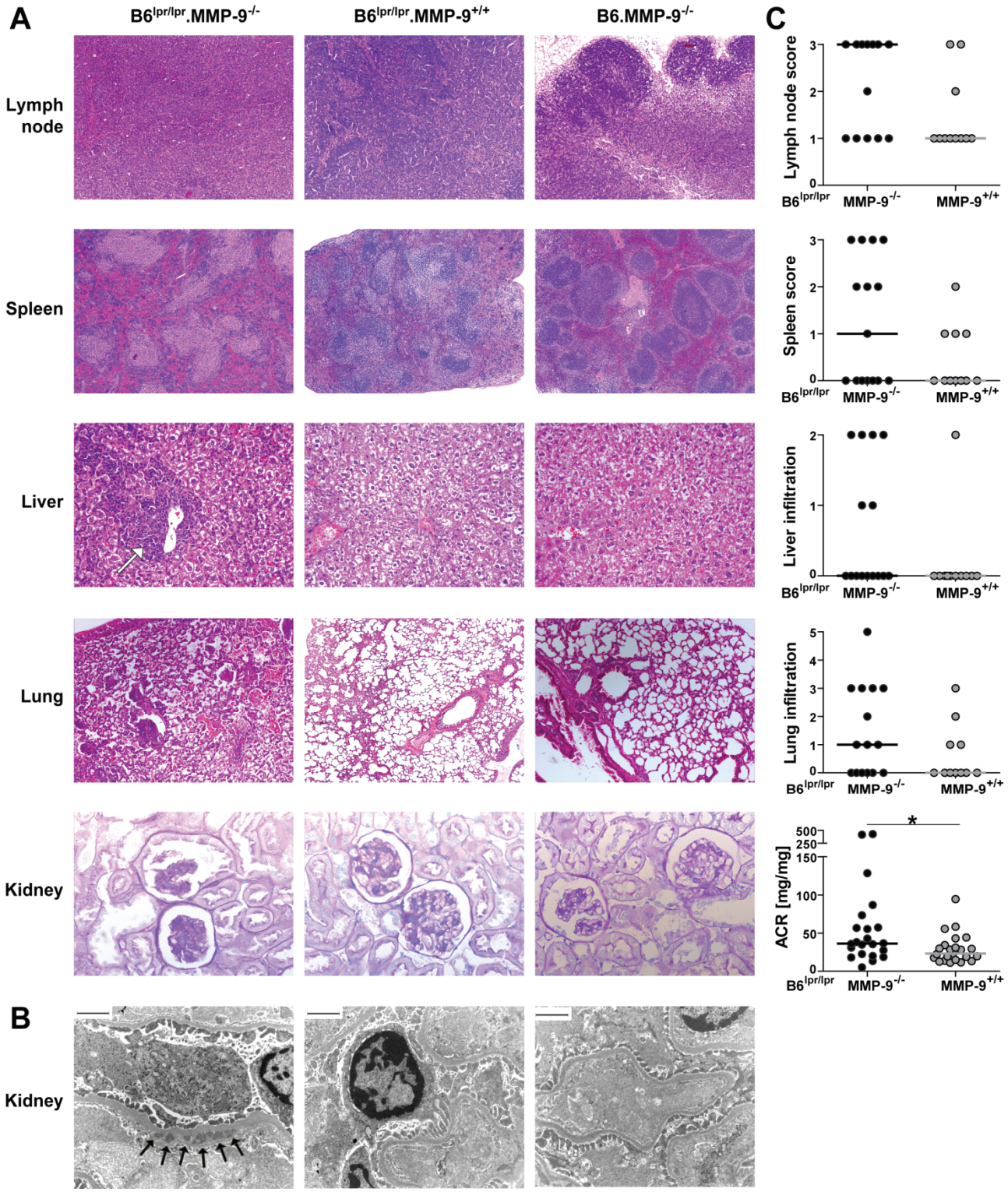

Figure 8. Autoimmune tissue injury is more pronounced in B6$^{lpr/lpr}$.MMP-9$^{-/-}$ mice *vs.* control mice. (A) Tissue sections from 9 months old mice were stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) stain (kidney sections) and scored by pathologists as detailed in 'Histologic examination of tissues'. Representative photomicrographs of axillary and inguinal lymph nodes (×100), spleen (×50), liver (×200), lung (×100) and kidney (×400) are shown for each genotype. White arrow, periportal infiltration. (B) Electron micrographs of glomerular capillaries in kidney samples prepared from frozen tissue blocs. Black arrows, immune complex deposits. Scale bars, 1.50 µm. (C) Histology scores and the albumin-to-creatinine ratio (ACR) for B6$^{lpr/lpr}$.MMP-9$^{-/-}$ and B6$^{lpr/lpr}$.MMP-9$^{+/+}$ mice. Horizontal lines indicate group medians. Each dot represents data from a single animal. *$P < .05$.

DISCUSSION

The Fas *lpr* mutation and the Fas ligand *gld* (generalized lymphoproliferative disease) mutation causing lymphoproliferation and lupus-like autoimmune symptoms in mice, were a key to the understanding of the autoimmune lymphoproliferative syndrome or ALPS etiology.[30] Patients with ALPS present with nonmalignant lymphoproliferation (splenomegaly and/or adenopathy) along with increase of unusual DN T cells, hypergammaglobulinemia and autoimmune disease, caused by heterozygous mutations in the Fas gene and concomitant defective lymphocyte apoptosis.[31] Similarly, it was discovered that also abnormal Fas expression was associated with expansion of cytokine-producing DN T cells.[32] The DN T cell population – which only represents 1% to 2% of peripheral T lymphocytes in healthy persons[33] – is also expanded in the peripheral blood of SLE patients and is characterized by increased levels of activation markers, production of IL-4, and induction of Ig and pathogenic anti-DNA autoantibodies.[34-37] Recently, IL-17-producing DN T cells were found in kidney biopsies of patients with lupus nephritis and were shown to induce nephritis in a non-autoimmune mouse model.[38,39]

Here, we describe the severe expansion of the DN T cell subset in the periphery of $B6^{lpr/lpr}$ mice rendered genetically deficient in the inflammatory protease gelatinase B/MMP-9. In the lymph nodes, DN T cell expansion was accompanied by increased accumulation of $CD4^+$ and $CD8^+$ T cells, B cells and myeloid cells such as neutrophils, macrophages and dendritic cells. The massive lymphoproliferation in $B6^{lpr/lpr}$.MMP-$9^{-/-}$ mice was positively correlated with higher autoantibody titers and more pronounced autoimmune injury with distortion of spleen and lymph nodes architectures, increased lymphocyte infiltration in non-lymphoid organs and enhanced kidney disease, culminating in increased mortality.

Various explanations may account for the described amplification of lymphoproliferation. On the one hand, the increased DN T cell expansion may be caused by an augmented proliferative capacity of MMP-9 deficient DN T cells *in vivo*, analogous to the increased alloreactive T cell proliferation observed in MMP-9 knockout mice after tracheal or cardiac allograft transplantation.[17,18] On the other hand, an additional apoptosis defect may be triggered by MMP-9 deficiency in immune cells, causing their increased accumulation in the periphery. In support of this, genetic lack of MMP-9 during zymosan peritonitis leads to enhanced cyclooxygenase-1 and prostaglandin E2 levels, which results in increased vascular permeability and decreased capase-3 expression and activity, the latter leading to impaired apoptosis and accumulation of inflammatory leukocytes.[40-42]

Deficient clearance of dying cells and cellular material is proposed to be a central pathogenic mechanism in the development and acceleration of systemic autoimmune diseases such as SLE.[16] Regarding the suppression of systemic autoimmune phenomena by MMP-9, it is tempting to hypothesize that MMP-9 deficiency engenders an additional clearance deficiency. Indeed, the massive load of accumulated DN T cells and other leukocytes that cannot undergo Fas-mediated apoptosis will eventually progress to necrosis - as evidenced by the hemorrhagic and necrotic appearance of many enlarged lymph nodes (Figure 2A) - and release intracellular autoantigens. MMP-9 was shown to degrade many intracellular proteins[12,13] and may in this way remove their immunogenic potential. Indeed, B cell conformational epitopes are likely to be destroyed by proteolysis, which was illustrated here by the impaired murine and human autoantibody binding to cleaved ribosomal protein P0 and cleaved U1snRNP A (Figure 7). In addition, intact polymeric substrate molecules such as actin and tubulin may crosslink B cell receptors and activate autoreactive B cells. Similarly, multivalent Toll-like receptor (TLR) ligands such as DNA and RNA may serve as co-activators or carriers for the internalization of bound antigens, and autoantigen-containing immune complexes may be internalized by autoreactive rheumatoid factor-positive B cells. Subsequently, B cell tolerance is broken by combined recognition of self-antigens and TLR activation. The autoantigens are processed and presented in MHC II molecules, and activate autoreactive T cells that provide help for other autoreactive B cells.[43] This phenomenon of 'B cell epitope spreading' may be suppressed by proteolysis of the (DNA/RNA-bound) antigen as more epitopes can be derived and presented from intact molecules as compared to residual carrier-bound fragments. This hypothesis was supported by the current observation that 'uncleaved' autoantigens seemed to be better stimuli for autoreactive B cells (Figures 5 to 7). However, further research is needed to define the exact impact of proteolysis on antigenicity, as the higher autoantibody titers in $B6^{lpr/lpr}$.MMP-$9^{-/-}$ mice may also result from an overall increase in systemic autoimmunity and epitope spreading to multiple autoantigens, both substrates and non-substrates. In this study, we have used MMP-9 as a model secreted protease, but it cannot be excluded that other 'inflammatory proteases' such as neutrophil elastase, granzyme B and cathepsins, might exert similar functions.

Upon initiation of the necrotic cascade, cells will release so-called damage-associated molecular patterns (DAMPs) or alarmins that mediate inflammation and immune cell activation.[44,45] Interestingly, many alarmins were found to be substrates of MMP-9, including high mobility group box 1/2 protein (HMGB1/2), HSP70, HSP90, hepatoma-derived growth factor, nucleolin and annexin I.[13] Proteolytic degradation of these pro-inflammatory adjuvant molecules may be a way of keeping them in check, as was described for HMGB1 cleavage by thrombin.[46] Along this line, activated dendritic cells were recently shown to be involved in autoimmune kidney injury in MRL$^{lpr/lpr}$ mice through secretion of HMGB1.[47] Hence, the higher numbers of activated dendritic cells combined with the lack of HMGB1 proteolysis in B6$^{lpr/}$lpr.MMP-9$^{-/-}$ mice may contribute to the observed enhancement of renal failure. As another point, low doses of self-peptides generated by proteolysis may promote tolerance by the induction of regulatory T cells and anti-inflammatory cytokines that suppress pathogenic T and B cells, as was shown for various peptides in murine lupus models.[48,49] Less regulatory T cells in the absence of MMP-9 may also be at the base of higher lymphocyte proliferation.

Since MMPs were shown to cleave a kaleidoscope of cell surface-associated molecules[13], proteolysis of such membrane molecules by MMP-9 may be an additional means of containing lymphocyte proliferation and systemic autoimmunity. For example, upregulation of the MMP-9 substrate intercellular adhesion molecule-1 (ICAM-1)[50,51] in correlation with disease activity was observed in patients with SLE and in MRL$^{lpr/lpr}$ mice.[52] Lack of ICAM-1 turnover by MMP-9 may contribute to the excessive leukocyte recruitment observed in B6$^{lpr/lpr}$.MMP-9$^{-/-}$ mice. Other interesting candidate substrates include the complement component 1 q subcomponent receptor 1 (C1qRp, CD93), FcγRIII (CD16), the IL-4 and IL-6 receptors, TNF receptor-55 and -75 and vascular cell adhesion molecule-1 (VCAM-1).[53]

In conclusion, this is the first time that genetic deficiency of a protease was described to enhance lymphoproliferation and systemic autoimmunity. As MMP-9 is an inducible protease, these effects were not visible under homeostatic conditions in MMP-9-deficient mice, but were revealed by the autoimmune lymphoproliferative genotype and phenotype of B6$^{lpr/lpr}$ mice. The enhanced pathology in B6$^{lpr/lpr}$.MMP-9$^{-/-}$ mice may be caused by a failure of various processes including disturbed lymphocyte homeostasis, apoptotic defects, clearance deficiencies and persistence of membrane-associated molecules. The ensemble of defects caused by MMP-9 deficiency in the B6$^{lpr/lpr}$ model is of clinical importance as our results suggest that long-term use of (non-selective) MMP inhibitors may be disease-promoting in ALPS and SLE patients, rather than disease-limiting.

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CONFLICT-OF-INTEREST DISCLOSURE

The authors declare no competing financial interests.

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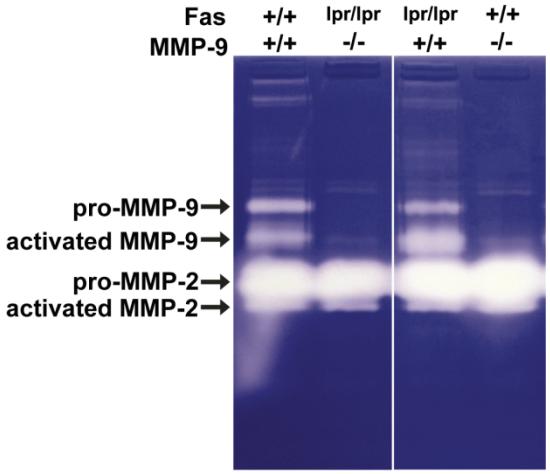
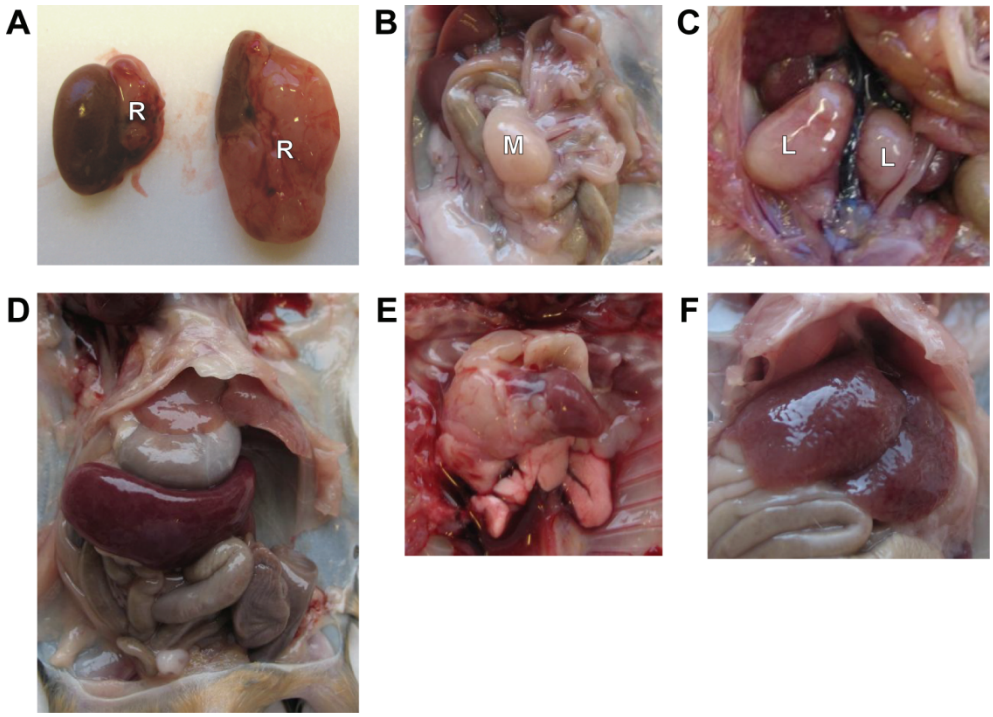
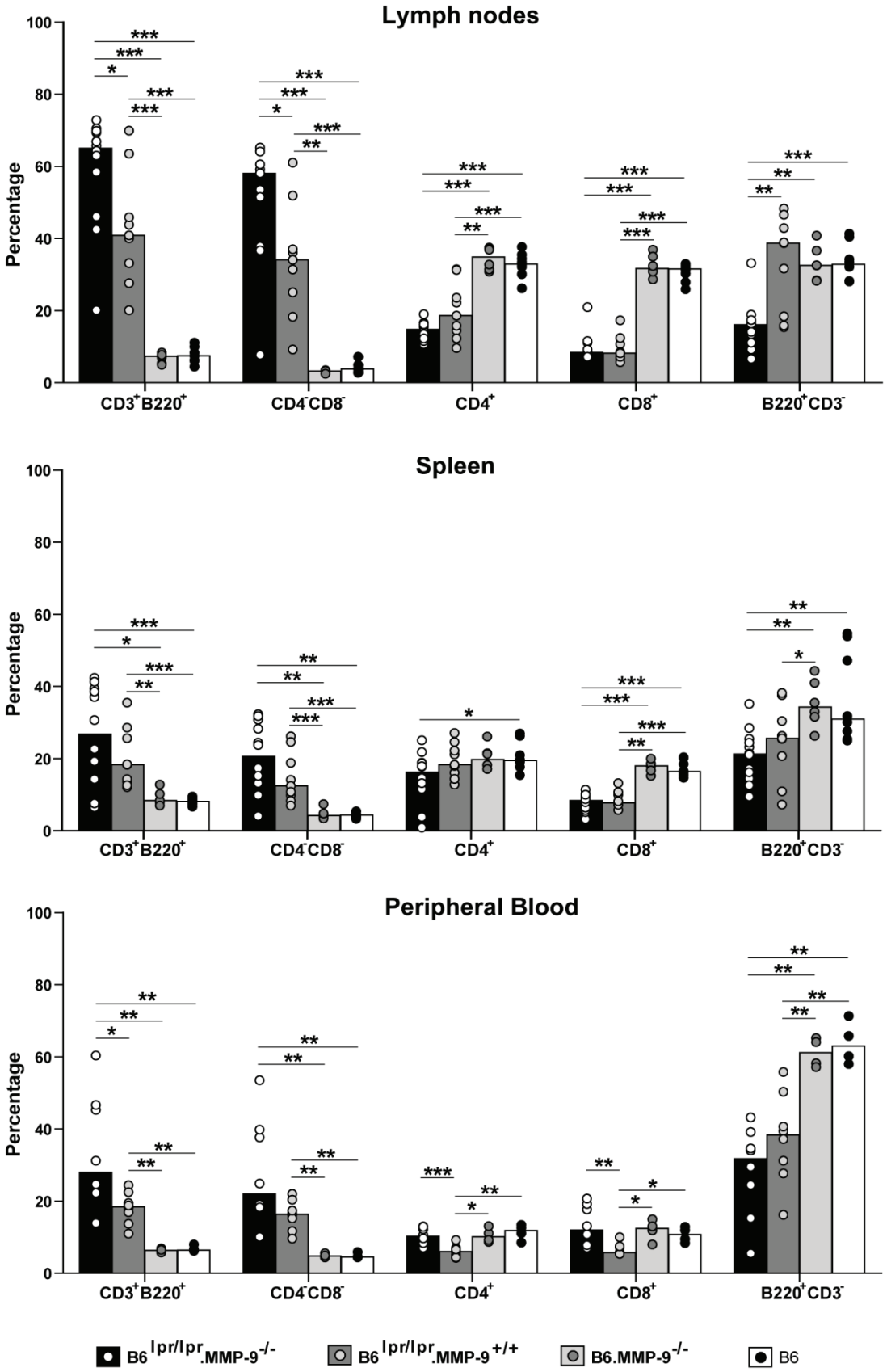
SUPPLEMENTARY MATERIAL



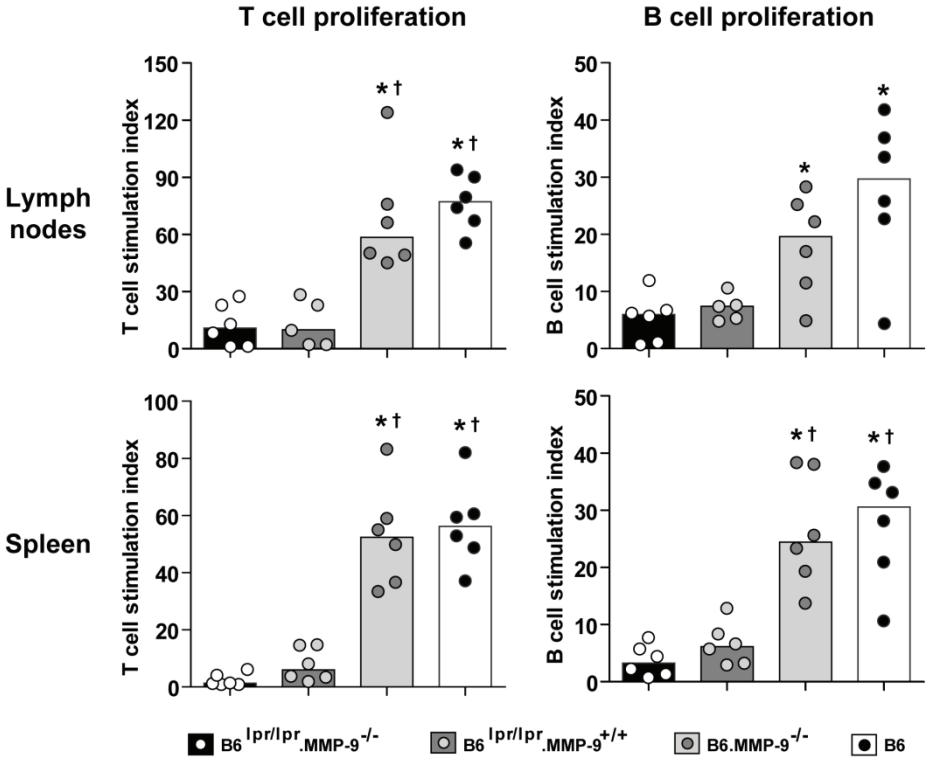
Supplementary figure 1. Phenotypic confirmation of genetic MMP-9 knockout by zymography analysis of plasma samples. Neutrophils in peripheral blood samples were triggered to degranulate for 1h by fMLP. Subsequently, gelatinases were prepurified from the plasma samples on gelatin-Sepharose and further analysed by gelatin zymography as described in 'Phenotyping'. The various gelatinase forms are indicated by arrows.



Supplementary figure 2. Increased macroscopic pathology in B6$^{lpr/lpr}$.MMP-9$^{-/-}$ mice. Enlarged (A) renal (R), (B) mesenteric (M) and (C) lumbal (L) lymph nodes in 6 to 9 months old MMP-9-deficient B6$^{lpr/lpr}$ mice. These mice often showed (D) splenomegaly, (E) an enlarged thymus and (F) a visibly affected liver with a granular surface and blunt edges.



Supplementary figure 3. Comparisons of lymphoid cell percentages in four genotype cohorts. Axillary and inguinal lymph node cells (top), splenocytes (middle) and peripheral blood cells (bottom) were isolated from 6 to 9 months old mice (age-matched groups) and stained as described in 'Materials and methods'. Double negative (DN) T cells were obtained by gating on $CD3^+B220^+$ cells followed by gating on $CD4^-CD8^-$ cells. Histograms show from left to right: percentages of $CD3^+B220^+$ cells, DN T cells ($CD4^-CD8^-$), $CD4^+$ T cells, $CD8^+$ T cells and $B220^+CD3^-$ B cells. Histograms indicate group medians and aligned scatter plots show the range of the individual data points. $*P < .05$, $**P < .01$, $***P < .001$.



Supplementary figure 4. No differences in T and B cell proliferation between B6$^{lpr/lpr}$.MMP-9$^{-/-}$ mice and B6$^{lpr/lpr}$.MMP-9$^{+/+}$ mice. Axillary and inguinal lymph nodes cells and splenocytes were isolated from 9 months old mice and stimulated to proliferate with anti-CD3 (as a polyclonal T cell stimulus) and anti-IgM (as an aspecfic B cell trigger). Proliferation was assessed by ^{3}H-thymidine uptake as detailed in 'Proliferation assays'. Histograms indicate group medians and each dot represents data from a single animal. **P* < .05, as compared with the B6$^{lpr/lpr}$.MMP-9$^{-/-}$ group. †*P* < .05, as compared with the B6$^{lpr/lpr}$.MMP-9$^{+/+}$ mice.

Supplementary Table 1. Correlations between macroscopic and microscopic pathology scores.

		Histopathology scores of affected organs			
	No.†	LN	Spleen	Liver	Lung
B6$^{lpr/lpr}$.MMP-9$^{-/-}$					
MPS	13	0,59 *	0,61 *	0,59 *	0,41
LN w/bw	13	0,84 ***	0,73 **	0,83 ***	0,45
Spleen w/bw	13	0,77 **	0,71 **	0,76 **	0,33
B6$^{lpr/lpr}$.MMP-9$^{+/+}$					
MPS	11	-0,13	-0,05	0,51	0,49
LN w/bw	11	-0,33	-0,19	0,50	0,52
Spleen w/bw	11	-0,59	-0,17	0,50	0,49

Depicted are Spearman correlation coefficients with their significance levels, which are indicated by **P* < .05, ***P* < .01, ****P* < .001.

†Number of pairs analysed by the Spearman rank correlation test.

LN, lymph nodes; MPS, macroscopic pathology score; w/bw, weight/body weight.

Supplementary Table 2. Correlations between autoantibody titers at 9 months and histopathology scores.

		Histopathology scores of affected organs			
Autoantibody specificity	No.†	**LN**	**Spleen**	**Liver**	**Lung**
B6$^{lpr/lpr}$.MMP-9$^{-/-}$					
anti-dsDNA IgG	13	0,39	0,39	0,49	-0,05
anti-chromatin IgG	11	0,05	-0,01	-0,06	-0,35
Rheumatoid factor IgG	13	0,29	0,23	0,50	0,45
anti-Histone IgG	13	0,54	0,65 *	0,68 **	0,23
anti-U1snRNP IgG	13	0,28	0,34	0,47	-0,01
anti-Smith IgG	13	0,62 *	0,67 *	0,62 *	0,21
anti-actin IgG	13	0,51	0,41	0,67 *	0,09
anti-tubulin IgG	13	0,41	0,45	0,63 *	0,29
B6$^{lpr/lpr}$.MMP-9$^{+/+}$					
anti-dsDNA IgG	10	-0,04	-0,07	0,52	0,27
anti-chromatin IgG	11	-0,33	-0,32	0,50	0,46
Rheumatoid factor IgG	11	-0,16	0,18	0,20	0,31
anti-Histone IgG	11	0,19	0,19	0,40	0,12
anti-U1snRNP IgG	10	0,02	-0,20	0,41	0,08
anti-Smith IgG	10	0,15	0,03	0,52	0,31
anti-actin IgG	10	0,17	0,02	0,52	0,49
anti-tubulin IgG	10	0,10	-0,66 *	0,41	0,21

Depicted are Spearman correlation coefficients with their significance levels, which are indicated by *P < .05, **P < .01.

†Number of pairs analysed by the Spearman rank correlation test.

LN, lymph nodes.

DISCUSSION AND PERSPECTIVES

In this section, a number of general findings of the present doctoral research are discussed in a wider context and as perspectives for future work. Part 1 consists of a review on the current status of intracellular substrate cleavage by MMPs. This part integrates our present ideas on intracellular MMP-9 substrates with findings obtained for other MMPs, and places the results of Chapters 2 and 3 in the framework of present knowledge on intracellular MMP substrates in physiology and pathology. Part 2 mainly deals with the conclusions of Chapter 4 in order to link these with the observations of Chapters 1, 2 and 3. In addition, it highlights the clinical relevance of further research on the roles of MMP-9 in systemic autoimmunity.

DISCUSSION PART 1. INTRACELLULAR SUBSTRATE CLEAVAGE: A NOVEL DIMENSION IN THE BIOCHEMISTRY, BIOLOGY AND PATHOLOGY OF MATRIX METALLOPROTEINASES

Cauwe B and Opdenakker G.

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INTRACELLULAR SUBSTRATE CLEAVAGE: A NOVEL DIMENSION IN THE BIOCHEMISTRY, BIOLOGY AND PATHOLOGY OF MATRIX METALLOPROTEINASES

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ABSTRACT

Matrix metalloproteinases (MMPs), originally discovered to function in the breakdown of extracellular matrix proteins, have gained the status of regulatory proteases in signaling events by liganding and processing hormones, cytokines, chemokines, adhesion molecules and other membrane receptors. However, MMPs also cleave intracellular substrates and have been demonstrated within cells in nuclear, mitochondrial, various vesicular and cytoplasmic compartments, including the cytoskeletal intracellular matrix. Unbiased high-throughput degradomics approaches have demonstrated that many intracellular proteins are cleaved by MMPs, including apoptotic regulators, signal transducers, molecular chaperones, cytoskeletal proteins, systemic autoantigens, enzymes in carbohydrate metabolism and protein biosynthesis, transcriptional and translational regulators, and proteins in charge of protein clearance such as lysosomal and ubiquitination enzymes. Besides proteolysis inside cells, intracellular proteins may also be modulated by MMPs in the extracellular milieu. Indeed, many intracellular proteins exit cells by non-classical secretion mechanisms or by various conditions of cell death by apoptosis, necrosis and NETosis, and become accessible to extracellular proteases. Intracellular substrate proteolysis by MMPs is involved in innate immune defense and apoptosis, and affects oncogenesis and pathology of cardiac, neurological, protein conformational and autoimmune diseases, including ischemia-reperfusion injury, cardiomyopathy, Parkinson's disease, cataract, multiple sclerosis and systemic lupus erythematosus. Since the same MMP may affect physiology and pathology in different and even opposite ways, depending on its extracellular or subcellular localization, an additional layer of complexity is added to therapeutic MMP inhibition. Hence, further elucidation of intracellular MMP localizations and intracellular substrate proteolysis is a new challenge in MMP research.

KEYWORDS: oxidative stress, apoptosis, cancer, innate immune defense, cardiopathology, neurodegeneration, autoimmunity, chaperone

ABBREVIATIONS:

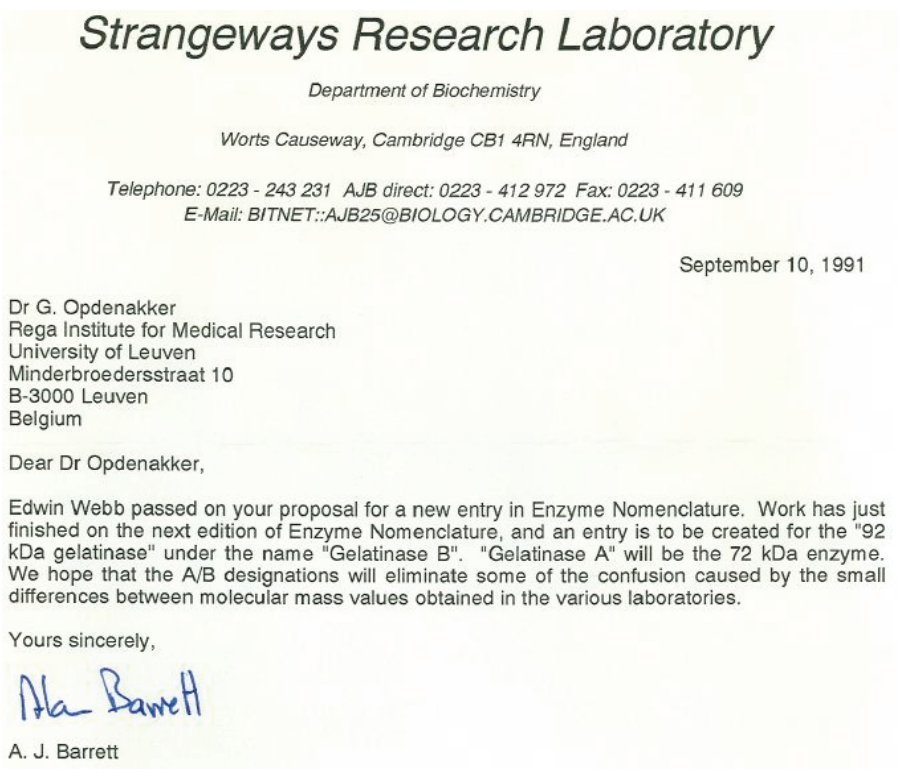
2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; **AMPA-R,** α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; **ATP,** adenosine triphosphate; **BBB,** blood-brain barrier; **CNS,** central nervous system; **DCM**, dilated cardiomyopathy; **ECM,** extracellular matrix; **ER,** endoplasmatic reticulum; **F-actin**, filamentous actin; **G-actin**, globular actin; **GSH**, reduced glutathione; **HMGB1/2**, High-mobility group box 1/2 protein; **hnRNP**, heterogenous nuclear ribonucleoprotein; **HSP**, heat shock protein; **I/R**, ischemia-reperfusion; **IFN-γ**, interferon-γ; **IL-1β,** interleukin-1β; **LPS**, lipopolysaccharide; **MI,** myocardial infarct; **MMP,** matrix metalloproteinase; **MS,** mass spectrometry; **MT-MMP,** membrane-type MMP; **NMDA-R1,** N-methyl-D-aspartate receptor-1; **O-phen**, 1,10-phenantroline; **PARP**, poly (ADP-ribose) polymerase; **PC,** proprotein convertase; **PD,** Parkinson's disease; **PMA**, phorbol 12-myristate 13-acetate; **PMN,** polymorphonuclear leukocytes; **RNS**, reactive nitrogen species; **ROS,** reactive oxygen species; **SDS**, sodium dodecyl sulphate; **SDS-PAGE,** sodium dodecyl sylphate-polyacrylamide gel electrophoresis; **TGN**, *trans*-Golgi network; **TIMP,** tissue inhibitor of metalloproteinases; **TJ,** tight junction; **TNF-α,** tumor necrosis factor-α.

TABLE OF CONTENTS

INTRODUCTION: FROM 'NOMEN EST OMEN' TO 'FORTUNA' OR 'HOW AND OMINOUS NAME MAY TURN OUT FORTUNATE'

The family of human matrix metalloproteinases (MMPs) currently consists of 24 different neutral endopeptidases. They play a role in many physiological processes such as reproduction, development, immune functions and tissue repair, but are also involved in pathological conditions such as cancer, inflammation, autoimmune diseases, vascular diseases and neurodegenerative disorders (Sternlicht and Werb, 2001; Egeblad and Werb, 2002; Manicone and McGuire, 2008; Milner and Cawston, 2005; Hu *et al.*, 2007; Rosenberg, 2009; Parks *et al.*, 2004).

Whether it is a good or bad attribute, the name of an enzyme needs to make any reader clairvoyant or interested in its activities. Originally, the enzymes that cleave extracellular matrix molecules (ECM) were named according to substrate conversion. For instance, collagenase was the name given to a collagen-degrading enzyme (Gross and Lapiere, 1962). When we purified a gelatin-degrading enzyme to homogeneity (Masure *et al.*, 1991), we were instructed by the IUPAC Enzyme Commission to name it gelatinase B, because of the used substrate and because a different gelatinase, henceforth named gelatinase A, was already defined (Figure 1). Along similar lines, the stromelysins, matrilysins and membrane-type enzymes were discovered and named. Stromelysin was, in fact, a better name given for a proteoglycan-degrading enzyme than proteoglycanase, because the latter name might wrongly suggest that the glycan structure (and not the protein) is cleaved in the matrix substrate molecule. At a certain time point, it was decided to rename these enzymes into matrix metalloproteinases. This nomenclature and abbreviation as MMP - with a suffix number - was based on the historic order of discovery. This renaming was not accomplished with perfect logics, since it was not decided to strictly adhere to one - e.g. the human – species. This led to unfortunate outcomes. For example, today MMP-4, MMP-5 and MMP-6 are not on the list anymore. The term metalloproteinase has remained a correct one, as all members have a catalytic Zn^{2+} ion in the active site. The addition matrix to metalloproteinase was correct in the original context of ECM substrates, but is recently contested by findings that these enzymes contribute also to cytokine, hormone and chemokine processing (Sternlicht and Werb, 2001; Parks *et al.*, 2004) and process many membrane-bound substrates (Cauwe *et al.*, 2007). In a recent review, Butler and Overall replaced matrix by multifaceted as a sign that the biology of these enzymes is gradually broadening (Butler and Overall, 2009b). In fact, the ominous nature of ECM-eating enzymes has indeed transcended to a higher order kind of proteases regulating biological control processes. In other words, simple soldiers have become generals and MMPs are now considered multifunctional entities. Unless the whole nomenclature system of these enzymes is reconsidered by means of delicate scientific diplomacy, the present adherence in more than 20.000 publications to this imperfect man-made classification has also fortunate side-effects. Indeed, as we will review here, MMPs cleave many "intracellular matrix" molecules (ICM). In fact, if we refer to matrix in its broadest sense, i.e. the organized grid or granular substance with multimolecular interactions, then the name given to these enzymes remains a tribute to many pioneers in the field and integrates, in an improved way, recent findings on the biology and pathology of MMPs.



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September 10, 1991

Dr G. Opdenakker
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Belgium

Dear Dr Opdenakker,

Edwin Webb passed on your proposal for a new entry in Enzyme Nomenclature. Work has just finished on the next edition of Enzyme Nomenclature, and an entry is to be created for the "92 kDa gelatinase" under the name "Gelatinase B". "Gelatinase A" will be the 72 kDa enzyme. We hope that the A/B designations will eliminate some of the confusion caused by the small differences between molecular mass values obtained in the various laboratories.

Yours sincerely,

A. J. Barrett

Figure 1

MMPs are multidomain enzymes characterised by a conserved three His Zn^{2+}-binding motif in the catalytic domain and a conserved Met turn following the active site (Bode *et al.*, 1993; Nagase and Woessner, 1999). The three His residues coordinate the Zn^{2+} ion, which interacts with a conserved Cys in the propeptide. This Cys-Zn^{2+} coordination confers latency to the MMPs which are synthesized as inactive pre-pro-enzymes (Visse and Nagase, 2003). The signal peptide is removed during translation, and activation of the pro-enzymes or zymogens requires disruption of the Cys-Zn^{2+} coordination (*cf.* Figure 3). The active site, now freed of the propeptide, binds substrates and the Zn^{2+} ion becomes available for the binding of a hydrolytic water

molecule that is essential for catalysis. Hence, the MMP activation mechanism was termed 'cysteine switch mechanism' (Van Wart and Birkedal-Hansen, 1990).

In terms of structure, a typical MMP consist of a propeptide, a catalytical domain, a Zn^{2+}-binding domain, a linker or hinge region of variable length, and a hemopexin domain, which contributes to substrate specificity and to interactions with endogenous inhibitors and cargo receptors (*cf.* Figure 3) (Piccard *et al.*, 2007). Exceptions to this multidomain organization rule are MMP-7/matrilysin-1, MMP-26/matrilysin-2 and MMP-23, which lack the hinge region and hemopexin domain. In addition, MMP-23/cysteine array (CA)-MMP has a unique Cys-rich domain and an immunoglobulin-like domain at the COOH-terminal side of the metalloproteinase domain. The structures of MMP-2/gelatinase A and MMP-9/gelatinase B contain three fibronectin type II repeats for the binding of gelatin, and MMP-9 is the only MMP to possess a Ser/Thr/Pro-rich O-glycosylated domain, which forms an anchorage site for multiple O-linked sugars (Van den Steen *et al.*, 2006). In addition to the secreted MMPs, six human membrane-bound MMPs (MT-MMPs) exist. These include four type I transmembrane proteins (MT1-MMP/MMP-14, MT2-MMP/MMP-15, MT3-MMP/MMP-16 and MT5-MMP/MMP-24) and two glycosyl phosphatidylinositol (GPI)-anchored proteins (MT4-MMP/MMP-17 and MT6-MMP/MMP-25) (Visse and Nagase, 2003; Nagase *et al.*, 2006).

As outlined in a number of pertinent review articles, MMP levels and activities are strictly controlled in a spatial and temporal fashion by genetic and epigenetic, by transcriptional, post-transcriptional and post-translational mechanisms, as well as by zymogen activation, receptor-mediated endocytosis and inhibition by natural inhibitors (Clark *et al.*, 2008; Emonard *et al.*, 2005; Ra and Parks, 2007; Nagase *et al.*, 2006; Brew and Nagase, 2010). The general protease inhibitor, α_2-macroglobulin, is the principal MMP inhibitor in the circulation (Baker *et al.*, 2002), whereas the tissue inhibitors of metalloproteinases (TIMPs) are considered to be the key inhibitors in tissue (Brew and Nagase, 2010).

A new challenge in MMP research is to (re)assess substrate repertoires. Logical but not so much applied ways to perform such assessments is by comparing and defining specific activities towards substrates, by in-depth biological studies to define the topologies of MMPs *in vivo* (Olson *et al.*, 2009; Sela-Passwell *et al.*, 2010) and to discover the substrates that are cleaved *in situ* (Agrawal *et al.*, 2006). The first twenty-five years of MMP research were successful by tedious purifications and characterizations of additional MMPs (Brinckerhoff and Matrisian, 2002; Nagase and Woessner, 1999; Nagase *et al.*, 1992).

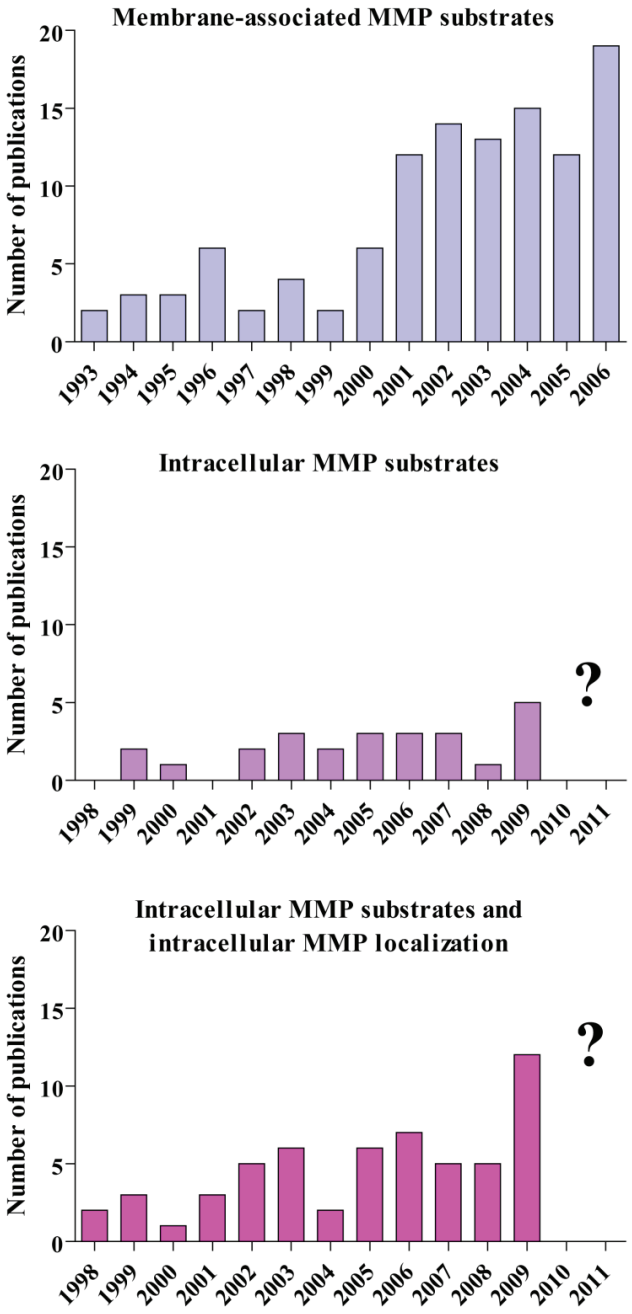

Figure 2. Comparison of the literature evolution for membrane-associated substrates *vs.* intracellular substrates. The graph of membrane-associated substrates is based on a previous review (Cauwe *et al.*, 2007). For literature on intracellular substrates and intracellular MMP localization, the Pubmed database was searched for combinations of matrix metalloproteinase with intracellular, subcellular, intranuclear, nucleus, intracellular substrate, intracellular cleavage and intracellular action key words. By comparison of the number of articles during the same number of years (>10 years after appearance of the first publication), it becomes clear that literature on intracellular substrates (middle graph) has not known the marked increase of membrane-associated substrate papers (top graph). However, an increase is noticed in the combination of data on intracellular MMP substrates and intracellular localization of MMPs. Hence, research on intracellular substrates and intracellular MMP actions is gaining interest and may catch up with other areas in MMP research.

The next milestone was the recognition that MMPs are not just massive ECM wreckers but also cleave many secreted molecules and membrane-associated substrates, which allows them to play sophisticated roles in the modulation of normal cellular behavior, cell-cell communication and tumor progression (McCawley and Matrisian, 2001; Sternlicht and Werb, 2001; Cauwe *et al.*, 2007). A dogma in MMP research has always been that they are secreted or membrane-associated proteases, acting in the extracellular space or at the cell surface. Many skeptical readers will acknowledge that true secretory enzymes such as MMPs have no business, physiologically speaking, cleaving substrates in a particular intracellular location. However, the presence of identified and functional subcellular localization signals in specific MMPs goes against this skepticism (Si-Tayeb *et al.*, 2006; Eguchi *et al.*, 2008). At a completely different level, it is easily accepted that MMPs may act in the extracellular milieu on many intracellular proteins, once they are actively or passively released from cells. Accumulating evidence suggests that the extracellular and pericellular actions may be complemented by intracellular actions, as well as by cleavage of intracellular proteins in the extracellular space. By comparison of the evolution of literature on membrane-associated substrates *vs.* intracellular substrates (Figure 2, top and middle graphs), it is quite obvious that studies on the cleavage of intracellular proteins by MMPs are lagging behind. Although a steady amount of articles have been published during the last ten years, a spectacular rise, as observed for membrane-associated substrates, has not yet been observed (Figure 2). Maybe, the idea of obligatory extracellular MMP action still hinders active investigation in this field and prevents that novel intracellular substrates are reported. However, when complementing publications on intracellular substrates with literature on intracellular MMP localization, one can see a rising trend, possibly of an opening field (Figure 2, bottom graph). The rationale of the present review is aimed at summarizing the current data on intracellular MMP localization and activation mechanisms. Furthermore, we will discuss present knowledge on the proteolysis of intracellular substrates by MMPs, both inside cells or in the extracellular milieu, and the concomitant physiopathological consequences of these cleavages. We hope that such an integrated view on intracellular substrate proteolysis will provide new insights and stimulate further research in this novel and exciting field of MMP research.

1. MECHANISMS OF EXTRACELLULAR *VS.* INTRACELLULAR MMP ACTIVATION

Disruption of the coordination between the catalytic Zn^{2+} ion and the conserved Cys in the propeptide is sufficient for latent MMPs to gain catalytic activity (Figure 3). The thiol-Zn^{2+} interaction may be broken by three mechanisms: 1) direct proteolysis of the propeptide by another MMP or protease; 2) modification of the free thiol by physiological reagents such as oxidants and disulfides, or by nonphysiological reagents such as alkylating agents and heavy metal ions; 3) distortion of the catalytic site by allosteric activation or by non-physiological reagents, including organomercurials (4-aminophenyl mercuric acetate or APMA), chaotropic agents (urea), and detergents (e.g. sodium dodecyl sulphate or SDS) (Ra and Parks, 2007; Sela-Passwell *et al.*, 2010; Springman *et al.*, 1990; Park *et al.*, 2010). The latter two mechanisms may allow MMPs to be activated by degradation of their own propeptide, which is called autocatalytic activation (Figure 3). Here, we discuss the physiological mechanisms that may lead to extracellular and intracellular MMP activation. Examples of intracellular activation mechanisms are summarized in Table 1.

1.1 Proteolytic cleavage

Historically, extracellular activation of pro-MMPs has been discovered mainly by in vitro studies with individual MMPs. The first examples were the activation of collagenase (MMP-1) by the serine protease plasmin (Werb *et al.*, 1977; Eeckhout and Vaes, 1977). These were seminal studies because they established a clear link between serine proteases and MMPs and reinforced the concept of enzyme cascades, as these were known for the complement and the coagulation cascades. Many individual studies became linked in a chain, called the ECM protease cascade (Cuzner and Opdenakker, 1999). By a critical analysis of the interrelations between the plasminogen activation system and MMP activations, it became clear that different MMPs can also activate each other. Hence, it was necessary to denominate all the interrelations as a complex network of interactions (Van den Steen *et al.*, 2002). This image of an activation network has evolved into a maze of dynamic protease interactions with other proteases, with inhibitors and with their substrates, which was termed the 'protease web' (Overall and Kleifeld, 2006) or the 'proteolytic internet' (Kruger, 2009).

Pei and Weiss were the first to discover a mechanism for intracellular MMP activation. They identified an Arg-X-Arg-X-Lys-Arg recognition motif for the Golgi-associated pro-hormone convertase furin between the pro- and catalytic domain of MMP-11/stromelysin-3 (Pei and Weiss, 1995). Furin cleaves MMP-11 behind this motif, resulting in an activated form of MMP-11 in the *trans*-Golgi network (TGN), which is subsequently secreted (Santavicca *et al.*, 1996). Likewise, pro-MT1-MMP is efficiently processed by furin and related proprotein convertases into an active protease after recognition of two basic motifs in the enzyme's prodomain (Pei and Weiss, 1996; Yana and Weiss, 2000). Of interest, such Arg-X-Lys-Arg motifs are not only found in MMP-11 and

MT1-MMP, but also in MMP-23 and in MT1-, MT2-, MT3-, MT4- and MT5-MMP. In addition, either Arg-X-X-Arg or Lys-X-X-Arg motifs are found in all MMPs except in MMP-12/metalloelastase and MMP-7. This suggests that a general mechanism exists for both intracellular and extracellular MMP activation by furin and proprotein convertases (Yana and Weiss, 2000). This was indeed confirmed for MT3-MMP, which co-localizes with furin in the TGN (Kang *et al.*, 2002). MMP-1, however, is not cleaved by furin, and MMP-2 is cleaved to an intermediate activation form, which is inactive. In contrast, MMP-3/stromelysin-1 cleavage by furin yields the anticipated molecular weight (MW) of active MMP-3, but its proteolytic activity was not verified (Cao *et al.*, 2005).

Furin-independent intracellular MMP activation modes also exist. For example, a serine protease different from furin generates an activated form of MMP-3 inside stressed dopaminergic cells (*vide infra*) (Choi *et al.*, 2008). Interestingly, in MMP-26 the latency motif containing the conserved Cys is inactive and MMP-26 is activated by autolytic cleavages (Marchenko *et al.*, 2002). In addition, the major fraction of synthesized MMP-26 remains intracellularly (Strongin, 2006) and may activate pro-MMP-9 in the cytoplasm (Uria and Lopez-Otin, 2000; Zhao *et al.*, 2003). Hence, the protease interaction network may also function inside cells. Indeed, MT1-MMP may be responsible for intracellular MMP-2 activation, since both MMPs were found to co-localize in the nuclei of aggressive hepatocellular carcinoma cells (Ip *et al.*, 2007) and in ischemic cell nuclei, which also contained co-localized furin (Yang *et al.*, 2010). A recent study showed that intracellular MMP activation may also be performed by caspases. Administration of activated caspase-3 to heart homogenates resulted in increased MMP activity. In addition, a cocktail of activated caspases generated activated forms of MMP-2, as detected by substrate zymography (Yarbrough *et al.*, 2010). However, whereas increased MMP activity in the heart homogenates was confirmed by cleavage of a fluorogenic substrate, this was not verified for the activated forms of MMP-2. This is an essential control, since alkylating agents such as dithiothreitol (DTT) preserve caspase stability but may activate MMPs by catalytic site distortion and autocatalysis.

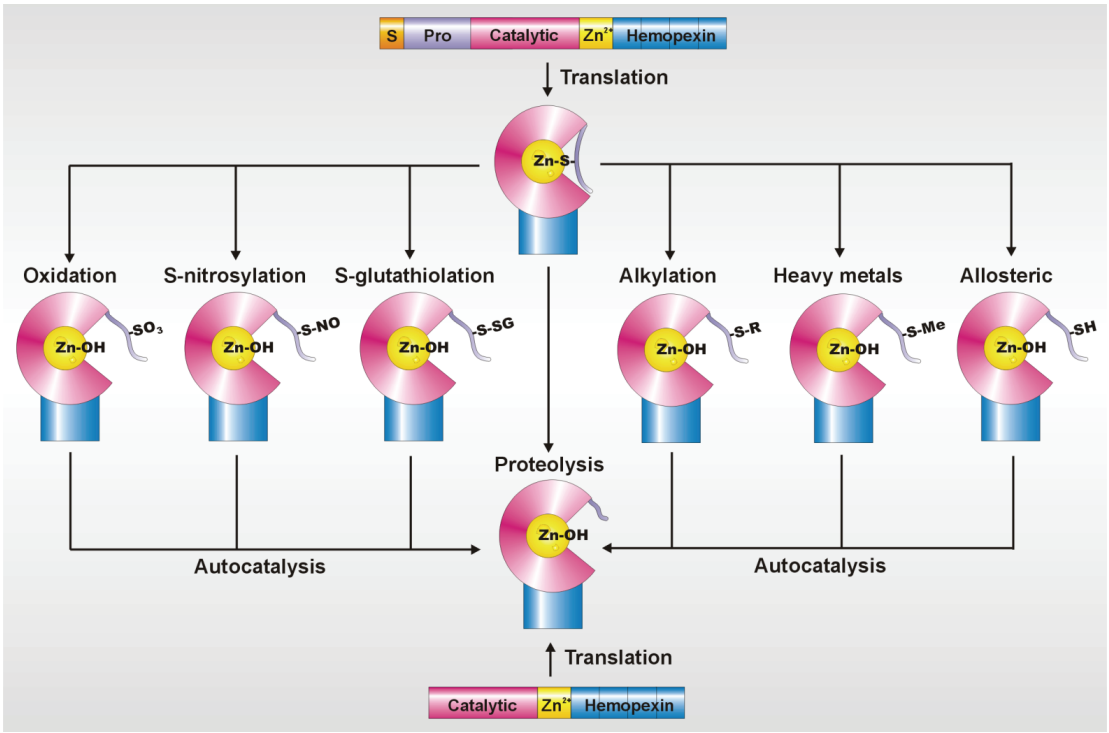

Figure 3. Activation mechanisms of MMPs. A typical MMP structure consists of a signal peptide (S), a pro-domain (Pro), a catalytic domain, a Zn^{2+}-binding domain (Zn^{2+}) and a hemopexin domain. MMPs gain catalytic activity by a mechanism called 'cysteine switch activation', in which the coordination between the catalytic Zn^{2+} ion and the conserved Cys in the propeptide is broken to open up the catalytic site for the binding of a hydrolytic water molecule and a substrate. The most common mechanism for the disruption of the thiol-Zn^{2+} coordination is the direct proteolysis of the propeptide by another MMP or protease. Other physiological activation pathways include the modification of the free thiol by physiological reagents such as oxidants (e.g. ROS) and disulfides (e.g. RNS), leading to oxidation, S-nitrosylation or S-glutathiolation of the Cys in the propeptide. Finally, distortion of the catalytic site by the binding of a receptor or a substrate may lead to allosteric activation. Non-physiological activation *in vitro* can be achieved by the modification of the Cys by alkylating agents and heavy metal ions (Me), and by the distortion of the catalytic site by non-physiological reagents, such as organomercurials (APMA), chaotropic agents (urea), and detergents (e.g. SDS). Non-proteolytic activation may lead to subsequent autoproteolytic degradation of the propeptide, which is called autocatalytic activation. Besides post-translational activation mechanisms, alternative splicing may yield MMP transcripts lacking the signal peptide and prodomain, which are translated into constitutively active enzymes.

1.2 Oxidative stress and nitrosative stress

In aerobic organisms reactive oxygen species (ROS) are constantly generated during normal metabolism and in response to both internal and external stimuli. Oxidative stress is caused by imbalances in the production and removal of ROS and is implicated in many pathological settings such as cancer, chronic inflammation, premature labor and stillbirth, ischemia/reperfusion (I/R) injury, atherosclerosis, arthritis and neurodegenerative disorders (Roberts *et al.*, 2009; Nelson and Melendez, 2004). ROS include many different chemical oxidants such as hydrogen peroxide (H_2O_2), superoxide anion ($^{\bullet}O2^-$), singlet oxygen ($^1O_2$), and hydroxyl radicals ($^{\bullet}OH$). Inside the cells, reactive oxygen species are constantly generated as side-products of neutrophil-mediated phagocytosis, by cellular respiration, and by various NADPH oxidases. Similarly, free radical nitric oxide ($NO^{\bullet}$) is a ubiquitous intracellular messenger able to regulate physiological functions (Martinez and Andriantsitohaina, 2009). However, when a disequilibrium occurs between the levels of $^{\bullet}O2^-$ and $NO^{\bullet}$, they may react to form peroxynitrite ($ONOO^-$), a reactive nitrogen species (RNS). Other RNS include $NO^{\bullet}$, nitroxyl (HNO), nitrosonium cation (NO^+), S-nitrosothiols (RSNOs) and nitrogen dioxide (NO_2). By analogy with ROS and oxidative stress, when the generation of RNS in a system exceeds its ability to neutralize and eliminate them, nitrosative stress occurs. In addition, cross-talk between ROS and RNS can exacerbate their capacity to damage DNA, lipids and proteins (Martinez-Pomares *et al.*, 1998; Brandes *et al.*, 2009; Martinez and Andriantsitohaina, 2009).

ROS may play important roles as signaling molecules, regulating many genes, including MMP gene expression and activity (Nelson and Melendez, 2004). Indeed, ROS interact with the Cys thiol, which disrupts the interaction with the catalytic Zn^{2+} ion and leads to autocatalytic activation (*cf.* Figure 3). Myeloperoxidase, a heme protein secreted by neutrophils, monocytes, and macrophages, uses hydrogen peroxide to generate hypochlorous acid (HOCl) (Hurst and Barrette, Jr., 1989). HOCl activates various pro-MMPs, including MMP-1, MMP-7, MMP-8, MMP-9 (Weiss *et al.*, 1985; Peppin and Weiss, 1986; Saari *et al.*, 1992; Fu *et al.*, 2001; Meli *et al.*, 2003). Whereas low levels of HOCl rapidly activate pro-MMP-7, higher concentrations or prolonged exposure inactivate the protease (Fu *et al.*, 2003). This biphasic effect was also observed in the activation of pro-MMP-2 by H_20_2, which also activates pro-MMP-8, pro-MMP-9, but not pro-MMP-1 and pro-MMP-7 (Saari *et al.*, 1992; Burkhardt *et al.*, 1986; Fu *et al.*, 2001; Rajagopalan *et al.*, 1996; Paquette *et al.*, 2003). Of interest, 30% of the MMP-8 released by synovial-fluid neutrophils in rheumatoid arthritis was in an activated form compared to the pro-MMP-8 form in peripheral blood neutrophils of the same patients (Saari *et al.*, 1992). This suggests that activation of MMP-8 may occur intracellularly before and/or during degranulation.

RNS induce three main post-translational modifications in proteins: 1) S-nitrosylation or the covalent addition of an $NO^{\bullet}$ group to a Cys thiol, 2) S-glutathiolation or the addition of reduced glutathione (GSH) or other low-MW thiols to a Cys thiol, and 3) tyrosine nitration or the addition of an NO_2 group to Tyr residues (Martinez and Andriantsitohaina, 2009). Hence, similar to ROS-mediated activation, RNS modification causes activation by disrupting the latency-inducing Cys-Zn^{2+} coordination (*cf.* Figure 3) (Ali and Schulz, 2009). Various pro-MMPs can be activated by S-nitrosylation *in vitro*, including MMP-1, MMP-2, MMP-8, MMP-9 (Rajagopalan *et al.*, 1996; Maeda *et al.*, 1998; Okamoto *et al.*, 1997; Gu *et al.*, 2002; Viappiani *et al.*, 2009). So far, activation by S-nitrosylation *in vivo* was only shown for MMP-9 during cerebral ischemia (Gu *et al.*, 2002). In addition, the nitrosylated thiol was oxidized further to a sulfinic ($-SO_2H$) or sulfonic ($-SO_3H$) acid. This irreversible modification may cause prolonged activation of the enzyme under pathological conditions and this progression represents a graded transition from physiological signaling functions to pathological nitrosative and oxidative stress, and finally, toxicity (Hess *et al.*, 2005). Activation by S-glutathiolation (with $ONOO^-$ and GSH) *in vitro* was demonstrated for pro-MMP-1, pro-MMP-2, pro-MMP-8 and pro-MMP-9 and it was more pronounced than activation with $ONOO^-$ alone (Okamoto *et al.*, 2001; Viappiani *et al.*, 2009). The high cellular concentrations of GSH under physiological conditions suppress the activation of pro-MMPs, whereas prolonged and sustained inflammation will result in high levels of pro-MMPs as well as ROS and RNS, which may tip the balance to glutathiolation and activation of MMPs (Okamoto *et al.*, 2001). The biphasic effect observed with oxidative activation (*vide supra*) is also detected with S-glutathiolation, suggesting that ROS and RNS may exert temporal control over MMP activity (Viappiani *et al.*, 2009). Of interest, RNS also inactivate TIMP-1, TIMP-2 and TIMP-4 (Frears *et al.*, 1996; Brown *et al.*, 2004; Chakraborti *et al.*, 2004; Donnini *et al.*, 2008), which further enhances MMP action. Surprisingly, both the groups of Schulz and Okamoto report nitrosative activation without the shift to the lower-MW activation form, expected after removal of the propeptide. This suggests that autocatalytic propeptide proteolysis does not always occur and that higher MW-forms may indeed be active. Hence, Schulz and coworkers state that the nomenclature of 'latent pro-enzymes' incorrectly assumes that only the lower-MW species are active (Ali and Schulz, 2009; Schulz, 2007). Here, we will refer to the high MW forms of MMPs as the pro-forms and avoid to use the terminology of 'latent' or 'inactive' forms, as these forms may be active in an oxidative context or even under physiological levels of ROS and RNS. The low MW forms (LMW) will be termed activated forms.

Table 1. (Putative) intracellular activation mechanisms of MMPs

Activation mode	Activator	pro-MMP	References
Proteolytic activation	furin/proprotein convertases	MMP-11 MT1-MMP MT3-MMP	(Pei and Weiss, 1996; Pei and Weiss, 1995; Yana and Weiss, 2000)
	serine protease	MMP-3	(Choi *et al.*, 2008)
	MMP-26 (autocatalysis)	MMP-26	(Marchenko *et al.*, 2002)
	MMP-26	MMP-9	(Uria and Lopez-Otin, 2000; Zhao *et al.*, 2003)
	MT1-MMP	MMP-2	(Ip *et al.*, 2007; Yang *et al.*, 2010)
	caspases	MMP-2	(Yarbrough *et al.*, 2010)
Oxidative stress	oxidation (HOCl)	MMP-1,-7,-8,-9	(Weiss *et al.*, 1985; Peppin and Weiss, 1986; Saari *et al.*, 1992; Fu *et al.*, 2001; Meli *et al.*, 2003)
	oxidation (H_2O_2)	MMP-2,-8,-9	(Saari *et al.*, 1992; Burkhardt *et al.*, 1986; Fu *et al.*, 2001; Rajagopalan *et al.*, 1996; Paquette *et al.*, 2003).
Nitrosative stress	S-nitrosylation ($ONOO^-$, NO_2, NO)	MMP-1,-2,-8,-9	(Rajagopalan *et al.*, 1996; Maeda *et al.*, 1998; Okamoto *et al.*, 1997; Gu *et al.*, 2002; Viappiani *et al.*, 2009)
	S-glutathiolation ($ONOO^-$, GSH)	MMP-1,-2,-8,-9	(Okamoto *et al.*, 2001; Viappiani *et al.*, 2009)
Phosphorylation	alkaline phosphatase	MMP-2	(Sariahmetoglu *et al.*, 2007)
Alternative splicing	no prodomain	MMP-11	(Luo *et al.*, 2002)

GSH, reduced glutathione; **H_2O_2**, hydrogen peroxide; **HOCl**, hypochloric acid;
NO, nitric oxide; **NO_2**, nitrogen dioxide; **$ONOO^-$,** peroxynitrite

In conclusion, modification of the conserved Cys can activate MMPs both outside and inside cells. The conservation of the Cys residue implies that these mechanisms may be applicable to most MMPs. Severe oxidative damage will ultimately lead to apoptosis and cell death, which has been shown to accompany many pathological conditions, such as cancer, cardiovascular diseases and neurodegenerative disorders (Martinez and Andriantsitohaina, 2009; Brandes *et al.*, 2009). These are all conditions in which intracellular MMP activities affect disease, which will be discussed in chapter 3.

1.3 Phosphorylation

Phosphorylation is a post-translational modification that typically regulates the activity of intracellular proteins and is of major importance for intracellular signal transduction and regulation of cellular function. The phosphorylation status of a protein is regulated by the balanced action of many protein kinases and phosphatases. Phosphorylation by protein kinase C diminishes MMP-2 activity *in vitro*, whereas dephosphorylation with alkaline phosphatase significantly enhances its activity (Sariahmetoglu *et al.*, 2007). MMP-2 contains 29 potential phosphorylation sites. All five sites that were phosphorylated in recombinant human MMP-2 purified from mammalian cells could be found on accessible residues at the surface of the protein. MMP-2 isolated from HT1080 cells showed phosphorylation of Thr, Ser and Tyr residues. Since most of the phosphorylation sites are within the fibronectin domain, which is essential for substrate binding, phosphorylation may also affect substrate affinity and specificity. Furthermore, phosphorylation may affect intracellular trafficking, protein stability and protein-protein interactions, as is the case for intracellular signaling and adaptor molecules (Sariahmetoglu *et al.*, 2007). Interestingly, MT1-MMP is also phosphorylated in its cytoplasmic domain, and impaired phosphorylation of Tyr573 inhibits tumor cell proliferation in three-dimensional matrices *in vitro* and tumor growth *in vivo* (Nyalendo *et al.*, 2008). Hence, investigation of the effects of phosphorylation on MMP activity and determination of the phosphorylation status of intracellular MMPs in homestatic conditions or under cellular stress, may yield information on the regulation of intracellular MMP activity.

1.4 Alternative splicing

Besides post-translational regulation, MMP activity may also be regulated by post-transcriptional regulation. Indeed, for MMP-11 an additional gene promoter was found that is inducible and controls the expression of a novel MMP-11 transcript in cultured cells and in placenta (Luo *et al.*, 2002). This transcript encodes a 40 kDa isoform that lacks both the signal peptide for

secretion and the prodomain (*cf.* Figure 3). As a consequence, a constitutively active isoform of MMP-11 is present in the intracellular milieu. This isoform is equally active as the secreted one but produced in 20-fold lower amounts. Surprisingly, human MMP-11 does not degrade the classical components of the ECM, unlike other MMPs and unlike mouse MMP-11 (Noel *et al.*, 1995). Remarkably, Luo *et al.* found a Met codon (for the initiation of a second transcript) within the DNA sequence of 10 other MMPs, suggesting that alternative splicing and promoter usage may be a characteristic of many MMPs. Indeed, splice variants lacking an NH_2-terminal secretory signal peptide have also been found for MMP-2 (e.g. accession number AL832088) (Sariahmetoglu *et al.*, 2007). In addition, a soluble *vs.* transmembrane form of MT3-MMP was formed by alternative splicing (Matsumoto *et al.*, 1997) and because MMP-23 lacks an NH_2-terminal signal peptide, it was also suggested to function intracellularly (Velasco *et al.*, 1999). Alternative transcripts have been found for various other MMPs and characterization of the associated isoforms may shed new light on intracellular locations and activities of many MMPs (Luo *et al.*, 2002).

2 INTRACELLULAR SUBSTRATE DETECTION BY DEGRADOMICS

Whereas proteomics is de study of the entire ensemble of proteins produced in a biological system or by an organism, degradomics aims at the characterization of the complete set of substrates, i.e. the repertoire or 'degradome' of a particular proteinase in a specific cell, tissue or organism (Lopez-Otin and Overall, 2002). During the last decade, many proteomic approaches have been adapted for degradomics and development of novel degradomics techniques has boomed. Since excellent and up-to-date reviews exist on this subject (Doucet *et al.*, 2008; Butler and Overall, 2009b; Doucet *et al.*, 2008; Morrison *et al.*, 2009; Rodriguez *et al.*, 2009; Demon *et al.*, 2009; Van Damme *et al.*, 2008; Agard and Wells, 2009; Diamond, 2007; Doucet and Overall, 2008; Overall and Blobel, 2007; Van Damme *et al.*, 2008; Impens *et al.*, 2010), we here describe briefly the various techniques along with their major advantages and drawbacks, and their application in MMP research (Table 2). In addition, we mention the major peptide-based techniques that were developed to characterize substrate cleavage sites, and provide additional references for the interested reader.

Various degradomics techniques were developed specifically for the identification of intracellular substrates and were applied for the study of particular caspase degradomes (Demon *et al.*, 2009; Van Damme *et al.*, 2008; Agard and Wells, 2009). These applications were intuitively developed on the basis that latent procaspases are activated and act within cells. Since the cascades of apoptosis, necrosis and netosis may lead to intracellular proteases entering the extracellular milieu, it is rarely questioned that typical extracellular substrates may end up in caspase degradomes. In contrast, although intracellular substrates have been defined as substrates of MMPs, both *in vitro* and *in vivo* (*vide infra*), the physiological or pathological relevance of such cleavages have only started to emerge. From Table 2, it is quite clear that many degradomics methods have not yet been used for the complete identification of MMP substrates. In addition, degradomics screens that identify *in vivo* substrates by direct comparison of wildtype and MMP knockout samples are still under-represented, which is probably also due to the lower reproducibility and high complexity of biological tissues and organ extracts.

In MMP research, degradomic approaches were aimed at identifying extracellular and membrane-bound substrates, and were used mainly to analyze secreted proteins in a cellular system or in 'secretomes' in the absence or presence of a particular MMP or MMP inhibitor. However, these diverging approaches always yielded many identifications of intracellular substrates, which were often not reported or discarded as 'background' from dying cells (Dean and Overall, 2007; Overall *et al.*, 2004; Tam *et al.*, 2004). Only very recently, these intracellular proteins started to be appreciated as a specific and novel subset of the MMP degradome (Butler and Overall, 2009b; Morrison *et al.*, 2009; Butler and Overall, 2009a; Cauwe *et al.*, 2008). Intracellular substrates may be released from cells during necrosis or by non-classical secretion mechanisms, which are often poorly characterized. Hence, many intracellular proteins may indeed end up and be cleaved by MMPs in the extracellular milieu. This setting will be discussed in detail in chapter 4.

In contrast with most degradomics methods applied for extracellular MMP substrate identification, a two-dimensional degradomics (2DD) approach was the first degradomics screen targeted directly at the identification of intracellular substrates. As illustrated in table 2, 2DD combines ion exchange chromatography (IEX) to separate proteins according to their isoelectric point (pI) before concentration and cleavage with a protease. Digested and undigested IEX fractions are then separated according to their molecular weight (MW) in adjacent SDS-PAGE lanes and differential protein bands are isolated for identification by MS/MS or electroblotted onto PVDF for identification by Edman degradation, which, in the case of a fragment band, results in direct identification of the cleavage site (Cauwe *et al.*, 2009). Using gelatinase B/MMP-9 as a model enzyme, we applied this method to THP-1 cytosol and isolated 100-200 differential proteins, of which about 70 intracellular proteins were identified as MMP-9 candidate substrates. Various advantages exist for this multidimensional degradomics method. First and foremost, this technically straightforward approach does not require complex and expensive equipment, making it broadly accessible and easy to start up in every laboratory.

Table 2. Degradomic approaches for the identification of MMP substrates and the determination of cleavage sites

Method	Short description	Advantages	Disadvantages	References	MMP studies	MMP	Type[1]
GEL-BASED DEGRADOMICS							
2D-PAGE (Two-dimensional polyacrylamide gel electrophoresis)	Proteolyzed and control protein mixtures are separated by two orthogonal electrophoresis steps: IEF according to pI and SDS-PAGE according to MW. Spots that disappear or decrease in abundance after cleavage are potential substrates. These may be picked out of the gel for identification by MS/MS after in-gel tryptic digest.	- decreases biological sample complexity *vs.* one-dimensional PAGE - identification of *in vivo* substrates - influence of PTMs on cleavage - inexpensive	- restricted to abundant and soluble proteins with average pI and MW - not quantitative: high intergel variability - labour intensive (many gels needed) - no cleavage sites identified	(O'Farrell, 1975; Friedman *et al.*, 2009)	(Hwang *et al.*, 2004; Descamps *et al.*, 2005; Hemers *et al.*, 2005; Sawicki *et al.*, 2005; Kim *et al.*, 2006)	MT1-MMP MMP-9 MMP-7 MMP-2	*In vitro* + *In vivo*
2D-DIGE (Two-dimensional difference gel electrophoresis)	Cleaved and uncleaved protein mixtures are labeled with different fluorescent labels, mixed and analyzed by 2D-PAGE. Fluorescence ratios of individual spots quantify degradation. The use of a mix of both samples as an internal standard, labeled with a third dye, allows for inter-gel comparisons.	- quantitative: intergel variability reduced by internal standard + higher sensitivity of fluorescent signals - identification of *in vivo* substrates - influence of PTMs on cleavage	- restricted to abundant and soluble proteins with average pI and MW - labour intensive - no cleavage sites identified	(Minden *et al.*, 2009; Unlu *et al.*, 1997)	(Greenlee *et al.*, 2006)	MMP-2 MMP-9	*In vivo*
Diagonal gel electrophoresis	A protein mixture is first separated according to MW by SDS-PAGE. Gel lanes are renaturated sepparately and subjected to in-gel proteolysis by an exogenously added protease. Gel lane proteins are subsequently separated by a second SDS-PAGE, perpendicular to the first, yielding the intact proteins on a diagonal and fragment spots under the diagonal.	- no protein precipitation by IEF - decreases biological sample complexity vs. one-dimensional PAGE - technically simple and inexpensive	- incomplete or incorect renaturation of substrates - limited access to substrates - restricted to abundant proteins - no cleavage sites nor *in vivo* substrates identified	(Nestler and Doseff, 1997)	ND	NA	NA
Shotgun proteomics	Proteolyzed and control protein mixtures are separated in adjacent SDS-PAGE lanes which are sliced into multiple bands. Proteins in each band are identified by LC-MS/MS after in-gel tryptic digests. Proteins are defined as substrates if the empirical MW displays a reduction by at least 20% compared with the theoretical MW, or if proteins are identified in clearly different gel slices.	- no protein precipitation by IEF - increased sensitivity compared to 2D-PAGE systems - identification of *in vivo* substrates	- complex bioinformatic and manual analysis of multiple MS/MS spectra - restricted to abundant proteins - only approximation of cleavages sites	(Thiede *et al.*, 2005)	ND	NA	NA
PROTOMAP (Protein topography and migration analysis platform)	Shotgun proteomics with visualization of protein-specific peptide coverage from all the individual slices in peptographs, which show the sequence coverage for a given protein on the X-axis (NH_2- to COOH-terminus) and the MW on the Y-axis (high to low MW)	- advantages of shotgun proteomics - facilitated and more quantitative data analysis than shotgun proteomics - comparison of unlimited number of samples, allowing for kinetic analysis	- restricted to abundant proteins - only approximation of cleavages sites	(Dix *et al.*, 2008)	ND	NA	NA

2DD (Two-dimensional degradomics)	A protein mixture is first separated by ion exchange chromatography (IEX) according to pI. All IEX fractions are concentrated and incubated in the absence or presence of a protease. Digested and undigested fractions are analyzed in adjacent SDS-PAGE lanes. Protein bands that disappear or decrease, and novel fragments that appear in the protease-digested fraction are potential substrates and identified by MS/MS after in-gel tryptic digests or by Edman degradation after electroblotting onto PVDF, which directly unveils the sequence of neo-NH_2-termini.	- reduced sample complexity by fractionation - no protein precipitation by IEF - lower abundancy substrates identified compared with other gel-based approaches - technically simple and inexpensive	- no *in vivo* substrates identified - labour intensive (many gels needed)	(Cauwe *et al.*, 2009)	(Cauwe *et al.*, 2009)	MMP-9	*In vitro*
DEGRADOMICS APPROACHES BASED ON QUANTITATIVE LABELING							
Reductive dimethylation	The proteolyzed and control sample are differentially labeled by reductive dimethylation with heavy (deuterium) or light (H) formaldehyde. Relative quantification of differentially mass-tagged proteins by MS/MS after trypsinization.	- technically simple and inexpensive	- high sample complexity by labeling of all free amines - no cleavage sites identified - high-end mass spectrometer needed	(Hsu *et al.*, 2003)	(Kleifeld *et al.*, 2010)	MMP-2	*In vitro*
$^{18}O/^{16}O$-labeling	The proteolyzed and control protein mixtures are trypsinized in the presence of ^{18}O water or natural ^{16}O water, which leads to differential incorporation of two light or two heavy ^{18}O's in the peptide COOH-termini.	- technically simple and inexpensive	- only two samples can be compaired - high sample complexity - no cleavage sites identified - high-end mass spectrometer needed	(Staes *et al.*, 2004)	ND	NA	*NA*
ICAT (Isotope-coded affinity tagging)	Cleaved and control protein mixtures are labeled with biotin-tagged reagents that differ in isotopic composition ($C^{13/12}$ or deuterium), pooled and digested with trypsin. Biotinylated peptides are pulled out and quantified by analysis of peak pairs in the MS spectra.	- quantification of relative protein abundancies - reduced sample complexity	- consumption of label by high abundancy proteins - proteins/peptides with few of no Cys will be missed - no cleavage sites identified - high-end mass spectrometer needed	(Gygi *et al.*, 1999)	(Butler *et al.*, 2008; Dean *et al.*, 2007; Dean and Overall, 2007; Tam *et al.*, 2004)	MT1-MMP	*In vitro*
iTRAQ™ (isobaric tags for relative and absolute quantitation)	Cleaved and control protein mixtures are trypsinized and all amino groups are labeled with iTRAQ™ labels that are chemically identical but fractionate differently during MS/MS, generating different spectral peaks. Relative quantification is obtained by peak-height analysis.	- more identifications than ICAT without Cys bias - identification of *in vivo* substrates - multiplexed quantification	- consumption of label by high abundancy proteins - expensive - no cleavage sites identified - no discrimination between cellular and serum proteins - high-end mass spectrometer needed	(Ross *et al.*, 2004)	(Dean and Overall, 2007)	MMP-2	*In vitro*
SILAC (Stable isotope labeling by amino acids in cell culture)	Duplicate cell cultures are grown in media containing stable isotope-labeled amino acids (e.g. ^{12}C- or ^{13}C-labeled Leu, Arg, Lys), subjected to a (cellular or exogenous) protease and subsequently pooled for MS/MS analysis. Relative quantification is obtained by peak-height comparison.	- labeling more efficient (100%) compared with ICAT and iTRAQ™ - discrimination between cellular and serum proteins - less errors by early pooling of samples	- no *in vivo* substrates identified - no cleavage sites identified - high-end mass spectrometer needed	(Ong *et al.*, 2002)	ND	NA	NA

SILAC MICE	Complete SILAC labeling of mice with a diet containing natural or ^{13}C-Lys over four generations.	- identification of *in vivo* substrates - *in vivo* kinetic analysis of cleavage	- time-consuming (breeding) - expensive - no cleavage sites identified - high-end mass spectrometer needed	(Kruger *et al.*, 2008)	ND	NA	NA
LABEL-FREE DEGRADOMIC APPROACHES							
2D LC-MS/MS (Two-dimensional liquid chromatography -MS/MS)	Proteolyzed and control protein samples are not pooled but analyzed separately by two independent rounds of liquid chromatography. Differences between cleaved and uncleaved samples are identified by spectral counting.	- reduced sample complexity - independent of labeling efficiency - inexpensive	- highly dependent on reproducibility because of separate sample analysis - time-consuming - favours high abundancy proteins - no cleavage sites identified	(Stoll *et al.*, 2007)	(Vaisar *et al.*, 2009)	MMP-9	*In vitro*
nano-UPLC-MS^E	Proteolyzed and control protein samples are not pooled but analyzed separately by RP-UPLC separation and coupled to MS/MS. MS data acquisitioin is achieved by the application of MS^E technology, which is based on continuous switching between low and high collision energy to collect precursor ion masses (low collision) and fragment ions (high collision) at ultrashort time intervals. Fragment ions are assigned to their corresponding precursor ion according to their similar retention times on ion chromatographs, which allows the creation of MS/MS spectra to obtain sequence and protein identifications.	- powerful high-troughput comparison of samples - collection of more precursor ions and fragmentation data by MS^E - independent of labeling efficiency - inexpensive - multiplexed quantification	- highly dependent on reproducibility because of separate sample analysis - complex data analysis - no cleavage sites identified - high-end mass spectrometer needed	(Xu *et al.*, 2008)	(Xu *et al.*, 2008)	MMP-9	*In vitro*
IDENTIFICATION OF NEO-NH_2-TERMINI							
COFRADIC (Combined fractional diagonal chromatography)	Negative selection is achieved by acetylating primary amines followed by trypsin digestion and capping of internal NH_2-peptides with TNBS, strongly increasing their hydrofobicity. Unmodified NH_2-terminal peptides elute earlier after RP-HPLC and are selected for MS analysis. Trypsin digestion in the presence of light ^{16}O or heavy ^{18}O water or SILAC labeling allow for quantification. By using trideutero-acetylation, the ε-amines of Lys and the *in vivo* free NH_2-terminal α-amines can be distinguished from *in vivo* acetylated NH_2-termini.	- powerful and quantitative high-throughput identification of substrates and cleavage sites - reduced sample complexitiy by neo-NH_2-termini renrichment - identification of *in vivo* substrates - cleavage sites identified	- inappropriate peptide lengths and poor ionizability limit the number of identifications - incomplete capture of internal peptides can lead to false positives - labour and equipment intensive - high-end mass spectrometer needed	(Gevaert *et al.*, 2003; Staes *et al.*, 2008; Van Damme *et al.*, 2005)	ND	NA	NA
Positional proteomics	Similar to COFRADIC but capture of internal peptides is achieved by incubation of the acetylated and trypsinized cell lysate with amine-reactive NHS-activated beads.	- fast and simple high-throughput identification of substrates and cleavage sites - reduced sample complexitiy by neo-NH_2-termini renrichment - identification of *in vivo* substrates - cleavage sites identified	- inappropriate peptide lengths and poor ionizability limit the number of identifications - incomplete capture of internal peptides can lead to false positives - not quantitative - high-end mass spectrometer	(McDonald *et al.*, 2005)	ND	NA	NA

			needed				
TAILS (Terminal amine isotopic labeling of substrates)	The proteolyzed and control sample are differentially labeled by reductive dimethylation with heavy or light formaldehyde, or by iTRAQ™ labeling, which labels all free NH_2-termini. Samples are mixed and trypsinized, generating internal peptides which are removed by a highly efficient high MW aldehyde-derivatized polymer. Remaining peptides are then analyzed by LC-MS/MS. May also be combined with SILAC labeling.	- powerful and fast high-throughput identification of substrates and cleavage sites - reduced sample complexitiy by neo-NH_2-termini renrichment - identification of cleavae sites and *in vivo* substrates - aldehyde polymer reduces false positives and improves sample recovery	- inappropriate peptide lengths and poor ionizability limit the number of identifications - incomplete capture of internal peptides can lead to false positives - aldehyde-derivatized polymer is not yet commercially available - high-end mass spectrometer needed	(Kleifeld *et al.*, 2010)	(Kleifeld *et al.*, 2010)	MMP-2	*In vitro*
Positive selection of neo-NH_2-termini	The use of O-methylisourea or subtiligase allows for selective biotinylation of NH_2-termini, which are captured by streptavidin beads and released chemically or enzymatically for LC-MS/MS analysis.	- increased sensitivity compared with negative selection methods by non-selection of endogenously acetylated NH_2-termini - reduced sample complexitiy by neo-NH_2-termini renrichment - identification of *in vivo* substrates - cleavage sites identified	- using O-methylisourea biotinylation of Ser, His, Thr can lead to false positives - subtiligase does not recognize Pro + low specificity for Glu and Asp - subtiligase labeling requires large amounts of material - no identification of cleavage before cyclized Gln, Cys and Glu - not quantitative - high-end mass spectrometer needed	(Timmer *et al.*, 2007; Mahrus *et al.*, 2008)	ND	NA	NA
INTERACTION-BASED DEGRADOMIC APPROACHES							
Exosite scanning	Recombinant MMP exosites are used to fish for interacting substrates in a proteome. This can be combined with a yeast two-hybrid screen or alternatively, domains that are used as 'bait' can be tagged to facilitate purification of the exosite and bound proteins for identification by MS/MS.	- greatly reduced sample complexity - additional information on non-catalytic domain functions	- not all interacting proteins are substrates - yeast two-hybrid is slow and yields many false positive and false negative results + only binary complexes identified - no cleavage sites nor *in vivo* substrates identified	(Overall *et al.*, 2002)	(McQuibban *et al.*, 2001)	MMP-2	*In vitro*
ICDC (Inactive catalytic domain capture)	A catalytically inactive domain mutant binds and captures substrates in a complex protein mixture. This can be combined with a yeast two-hybrid screen or alternatively, the inactive catalytic domains can be tagged to facilitate purification of the protein complex for identification by MS/MS.	- greatly reduced sample complexity	- yeast two-hybrid is slow and yields many false positive and false negative results + only binary complexes identified - no cleavage sites nor *in vivo* substrates identified	(Overall *et al.*, 2004)	(Overall *et al.*, 2004)	MT1-MMP	*In vitro*

PEPTIDE LIBRARY-BASED CHARACTERISATION OF SUBSTRATE AND CLEAVAGE SITE SPECIFICITIES							
Phage display substrate	Phages displaying random peptides are fused to a ligand and immobilized on an affinity support through a receptor, After treatment with a protease, substrate peptides are cleaved, releasing the concordant phages from the solid phase, which are then used to infect F-positive bacteria to be amplified for a next selection step. After several rounds of selection, phages are cloned and DNA of the individual phages is amplified to identify the cleavable peptides.	- systematic characterisation of substrate cleavage	- no cleavage of the native substrate conformation - labour intensive - limited number of identifications - no cleavage sites nor *in vivo* substrates identified	(Smith, 1985; Deperthes, 2002)	(Smith *et al.*, 1995; Deng *et al.*, 2000; Chen *et al.*, 2002; Kridel *et al.*, 2001; Ohkubo *et al.*, 2001)	MMP-3 MMP-7 MMP-13 MMP-2 MMP-9 MT1-MMP	*In vitro*
CLiPS (Cellular libraries of peptide substrates)	Recombinant peptides bound to fluorescent-probe peptide ligand are displayed on the surface of bacterial cells. Substrate peptide cleavage results in a reduction of cellular fluorescence, which is detected by flow cytometry. Non-fluorescent cells are removed and the enrichment of clones with hydrolyzed substrates is repeated.	- systematic characterisation of substrate cleavage - quantitative real-time measurement - influence of PTMs on cleavage	- no cleavage of the native substrate conformation - labour intensive - limited number of identifications - no cleavage sites nor *in vivo* substrates identified	(Boulware and Daugherty, 2006)	ND	NA	NA
mRNA display	Starting from a cDNA library, an mRNA transcript is ligated at its 3' with puromycin. Translation of the puromycin-linked mRNA results in a mRNA-puromycin-peptide fusion product, of which the mRNA is made double-stranded by RT-PCR. The peptide is biotinylated at its NH_2-terminus and immobilized on streptavidin beads. Cleavage by a protease releases the DNA-puromycin-peptide fusion complex and DNA is amplified by PCR, enriching the library with substrate sequences.	- systematic characterisation of substrate cleavage	- no cleavage of the native substrate conformation - labour intensive - limited number of identifications - no cleavage sites nor *in vivo* substrates identified	(Ju *et al.*, 2007)	ND	NA	NA
PS-SCL (Positional scanning synthetic combinatorial libraries)	Positional scanning libraries are generated by keeping a single position in a peptide constant, while other peptides are diversified with all possible combinations of amino acids, typically excluding cysteine. Cleavage is detected by release of a fluorescent leaving group from the peptide or by removal of a quenching group.	- systematic characterisation of substrate cleavage and cleavage sites	- no cleavage of the native substrate conformation - labour intensive - limited number of identifications - no in vivo substrates identified	(Lam and Lebl, 1998; Backes *et al.*, 2000; Diamond, 2007)	(McGeehan *et al.*, 1994)	MMP-1 MMP-9	*In vitro*
PICS (Proteomic identification of protease cleavage sites)	Proteome-derived libraries are generated by digestion with trypsin, GluC or chymotrypsin. Sulfhydryl groups are then protected with iodacetamide and primary amines are blocked by reducted methylation into tertiary amines. The resulting peptide mixture is incubated with a protease and neo-NH_2-terminal peptides are biotinylated and pulled out by immobilized streptavidin. These peptides are sequenced by LC-MS/MS, yielding the prime-side[2] sequence of the cleavage site.	- fast and robust profiling of protease-active site specificities and subset cooperativity - cleavage sites identified	- no cleavage of the native substrate conformation - modification of all Cys and Lys residues may interfere with peptide-protease interaction - no *in vivo* substrates identified	(Schilling and Overall, 2008)	(Schilling and Overall, 2008)	MMP-2	*In vitro*

Legend Table 2. **IEF,** isoelectric focusing; **LC-MS/MS**, liquid chromatography followed by tandem mass spectrometry; **MS**, mass spectrometry; **MSE**, high/low collision energy MS; **MW**, molecular weight; **NA,** not applicable; **ND,** not defined; **NHS,** N-hydroxysuccinimide; **pI,** isoelectric point; **PTM**, post-translational modification; **PVDF,** polyvinylidene fluoride; **RP-HPLC,** reversed phase-high pressure liquid chromatography; **RT-PCR,** real-time PCR; **SDS-PAGE,** sodium dodecyl sulphate-polyacrylamide gel electrophoresis; **TNBS**, 2,4,6-trinitrobenzenesulfonyl chloride; **UPLC,** ultraperformance liquid chromatography.

[1]Substrate type, describes whether the MMP substrates found in the described degradomics screen are *in vitro* or *in vivo* substrates.

[2]The substrate amino acids surrounding a cleavage site are indicated as Pn…P3-P2-P1 ↓ P1'-P2'-P3'…Pn', with the cleavage occurring at the peptide bond between P1 and P1' (Berger and Schechter, 1970).

Although the generation of multiple IEX fractions and SDS-PAGE gels may be somewhat labor-intensive, this is counterbalanced by the fact that final results can be obtained within a few weeks from start-up, as there is no need for lengthy and extensive optimization procedures as in most complex degradomic approaches. Second, the high loading capacity of the IEX columns and the insertion of a concentration step increase the dynamic range. This allows for the identification of both high and low abundancy class substrates. Third, as the protein pool is fractionated before proteolysis, less saturation of the protease by high abundancy class proteins will occur during digestion. Finally, 2DD can be easily extended to any protease, protease inhibitors may be applied to block specific enzymes and the technique is applicable with any cell type, tissue extract or body fluid. A selection of 2DD candidate substrates was confirmed biochemically by *in vitro* cleavage of the purified or recombinant substrate and visualization of cleavage by classical SDS-PAGE analysis. Alternatively, THP-1 cytoplasm was digested with MMP-9 *in vitro* and cleavage of specific substrates was confirmed by Western blot analysis of cleaved and uncleaved cytoplasm with substrate-specific primary antibodies. The collection of identified cleavage sites allowed us to analyze the cleavage site preference of MMP-9 in intracellular substrates and to compare this with observed preferences for extracellular and membrane-bound molecules. This was achieved by the generation of a sequence logo, using the bioinformatics software WebLogo 3, which adjusts for small samples and corrects for natural amino acid abundances (Crooks *et al.*, 2004; Schneider and Stephens, 1990) (Figure 4). This sequence logo clearly shows similarities between extracellular and intracellular MMP substrates.

When analyzing the biological functions of the intracellular MMP-9 (candidate) subtrates, we found that about 40% of these proteins were components of the cytoskeleton or intracellular matrix (ICM), or associated with ICM proteins (Cauwe *et al.*, 2009). In addition, two thirds of the candidates were autoantigens in cancer or in (multiple) autoimmune diseases. In order to gain insight into the diversity of intracellular proteins cleaved by all MMPs, we scrutinized the results of the MMP degradomics studies mentioned in Table 2 and compiled a general list of high-confidence degradomic candidate substrates (Table 3). Addition of a substrate to the list was based on the identification in various degradomics approaches, the (putative) cleavage by more than one MMP, the identification of a protein of the same subfamiliy or with a similar function as an MMP (candidate) substrate or the biochemical confirmation by *in vitro* cleavage or Western blot analysis (*vide supra*). This yielded a table of more than 120 proteins that were classified according to functional biological mechanisms. From Table 3 it is clear that cytoskeletal or ICM proteins constitute a considerable fraction of the intracellular degradome. This suggests that degradation of the ICM is a general MMP function. Other over-represented protein classes are carbohydrate metabolic and protein biosynthetic enzymes, proteins regulating transcription and translation, and molecular chaperones. The (putative) physiopathological contexts engendered by the cleavage of these intracellular proteins will be discussed throughout the manuscript. The leading theme is that such cleavages do take place and, irrespectively whether these are causes or consequences in physiological or pathological processes, they need to be considered if we critically (re)consider disease processes.

The fact that very diverging degradomics approaches, ranging from gel-based approaches to complex isotope- and MS-based methodologies converge to a common list of substrates, is not only an internal validation of these methods, but also a confirmation that these biochemical identifications are not just 'background noise' and need to be considered as meaningful in pathophysiological processes. The major advantages of degradomics indeed lie in the unbiased and high-throughput nature, which allows to obtain novel and unexpected views on the MMP degradome. Hence, expanding the application of MMP degradomics is of utmost importance and may yield many surprising insights and add novel dimensions to the kaleidoscope of MMP functions (Cauwe *et al.*, 2007; Butler and Overall, 2009b).

3 MMP ACTION INSIDE CELLS

Intracellular activation of MMPs and presence of activated MMPs in various intracellular compartments strongly suggests that MMPs may be responsible for proteolytic actions on intracellular substrates within the cells. In this chapter, we will first discuss how intracellular MMP activity can be detected. Next, we will summarize the present information concerning the presence of activated MMPs in various subcellular locations and discuss the mechanisms by which MMPs (may) enter cells and translocate to specific cellular compartments. Finally, we will discuss the physiological and pathological effects of intracellular cleavage by MMPs in innate immune defense, cancer, cardiac and brain disease, cataract development and in the process of apoptosis.

Table 3. Confirmed and high-confidence* intracellular MMP substrates identified by degradomics

Protein name	MMP	Method‡	Confirm.‡	Subcellular localization	(Putative) function	Refs‡
CARBOHYDRATE METABOLISM						
Citrate synthase	MMP-2 MMP-9	iTRAQ™ 2DD	ND **IVC**	mitochondrial matrix	Tricarboxylic acid cycle enzyme	5,10
Enolase-α	MMP-2 MMP-9	iTRAQ™, TAILS 2DD	ND	cytoplasm, cell membrane, myofibril, sarcomere, M-band, nucleus, extracellular	Glycolytic enzyme; role in growth control, hypoxia tolerance and allergic responses; receptor and activator of plasminogen	5,10,11
Enolase-β	MMP-2 MT1-MMP	TAILS ICAT	ND	cytoplasm, sarcomere, Z-band	Glycolytic enzyme; function in striated muscle development and regeneration; defects are the cause of glycogen storage disease type 13	8,11
Enolase-γ	MMP-2 MMP-1,-8,-9 MT1-MMP	ICAT, TAILS ICAT	**IVC**	cytoplasm, cell membrane, extracellular	Glycolytic enzyme; has neurotrophic and neuroprotective properties	6,8,11
Fructose-bisphophate aldolase A	MMP-2 MT1-MMP MMP-9	iTRAQ™, ICAT, TAILS ICAT UPLC-MSE, 2DD	ND	cytoplasm, cytoskeleton, I-band, extracellular	Glycolytic enzyme that catalyzes the reversible conversion of fructose-1,6-bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate; actin filament organization; defects are the cause of glycogen storage disease type 12	5,6, 7,8, 10,11
Fructose-bisphophate aldolase C	MMP-2	ICAT	ND	cytoplasm, cytoskeleton	Glycolytic enzyme that catalyzes the reversible conversion of fructose-1,6-bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate	6
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	MMP-2 MT1-MMP MMP-9	iTRAQ™, ICAT, TAILS ICAT UPLC-MS^E	ND	cytoplasm, cell membrane, extracellular	Glycolytic enzyme; independent of its glycolytic activity it is also involved in membrane trafficking in the early secretory pathway.	5,6,7, 8,11
Malate dehydrogenase	MMP-2 MT1-MMP	iTRAQ™, ICAT, TAILS ICAT	ND	cytoplasm, extracellular	Tricarboxylic acid cycle enzyme	5,6, 8,11
Phosphoglycerate kinase 1	MMP-2 MT1-MMP MMP-9	ICAT, TAILS ICAT UPLC-MSE, 2DD	ND	cytoplasm, extracellular	Glycolytic enzyme; putative polymerase α cofactor protein: defects are associated with chronic hemolytic anemia	5,6, 7,8, 10
Phosphoglycerate mutase 1	MMP-2	ICAT, TAILS	ND	cytoplasm, extracellular	Glycolytic enzyme; interconversion of 3- and 2-phosphoglycerate with 2,3-bisphosphoglycerate as the primer of the reaction	6,11
Triose phosphate isomerase	MMP-2 MT1-MMP MMP-9	iTRAQ™, ICAT, TAILS ICAT UPLC-MS^E	ND	cytoplasm, extracellular	Enzyme of the glycolytic and gluconeogenesis pathways; defects are the cause of triosephosphate isomerase deficiency	5,6, 7,8, 11
CYTOSKELETON						
Actin-β/γ	MMP-2 MMP-9 MMP-11 MMP-1,-8,-13	TAILS UPLC-MSE, 2DD TAILS IVC	**WB** **IVC, WB** ND **WB**	cytoplasm, cytoskeleton, nucleus, cell projection, extracellular	Vital for cell morphogenesis and motility, endocytosis, phagocytosis and cytokinesis	7,10,11
α-Actinin-1	MMP-2 MT1-MMP MMP-9	ICAT, iTRAQ™ ICAT UPLC-MS^E	**(Sung *et al.*, 2007)**	cytoskeleton, myofibril, sarcomere, Z-disc, nucleolus, extracellular	Connection of actin filaments of adjacents sarcomeres and transmission of the force generated by the actin-myosin complex	5,6, 7,8
α-Actinin-4	MMP-2 MT1-MMP MMP-9	TAILS ICAT UPLC-MS^E	ND	cytoplasm, cytoskeleton, cell projection, nucleolus, extracellular	Connection of actin filaments of adjacent sarcomeres and transmission of the force generated by the actin-myosin complex; defects are the cause of focal segmental glomerulosclerosis	7,8,11
Actin regulatory protein CAP-G	MMP-2 MT1-MMP MMP-9	iTRAQ™, ICAT ICAT 2DD	ND	cytoplasm, melanosome, nucleus, secreted	Reversible blocking of actin filament barbed ends without filament severing; may have important roles in macrophage function and regulation of cytoplasmic and/or nuclear structures through interactions with actin; potential DNA binding	6,8, 10
Actin-related protein (Arp) 2	MMP-9 MT1-MMP	2DD ICAT	**IVC** ND	cytoplasm, cytoskeleton, cell projection	ATP-binding component of the Arp2/3 complex which mediates the formation of branched F-actin networks	8,10

Actin-related protein (Arp) 2/3 complex subunits	MMP-9 MMP-1,-2,-3,-8,-13	2DD IVC	**IVC**	cytoplasm, cytoskeleton, cell projection	Actin-binding component of the Arp2/3 complex which mediates the formation of branched F-actin networks	8,10
Adenylyl cyclase-associated protein-1 (CAP1)	MMP-2,-8,-13 MMP-9	IVC 2DD	**(Cauwe *et al.*, 2008)**	cytoplasm, cell membrane	Enhancement of actin filament turnover; roles in cell morphology, migration and endocytosis; promotion of cofilin-induced apoptosis by shuttling actin to mitochondria	10
Cofilin-1	MMP-2 MT1-MMP	ICAT, TAILS ICAT	ND	cytoplasm, cytoskeleton, nuclear matrix, extracellular	Actin filament depolymerization and severing protein. Promotion of apoptosis by translocating to mitochondria and delivering actin	6,8, 11
Desmin	MMP-2	TAILS	**(Sung *et al.*, 2007)**	cytoplasm, cytoskeleton, Z-disk	Intermediate filament protein involved in cellular resistance to external stress	11
Ezrin	MMP-2 MT1-MMP MMP-9	iTRAQ™ ICAT 2DD	ND	cytoplasm, cell membrane, cell projections, extracellular	Cross-linking of cortical actin filaments and plasma membranes; roles in ECM interactions, cell-cell communication, apoptosis, carcinogenesis and metastasis	5,8, 10
Fascin	MMP-9	2DD	ND	cytoplasm, cytoskeleton, cell membrane	Organization of F-actin into bundles with a minimum of 4.1:1 actin/fascin ratio	10
Filamin A	MMP-2 MT1-MMP	iTRAQ™, ICAT, TAILS ICAT	ND	cytoplasm, cytoskeleton, nucleus, cell membrane, extracellular	Promotion of actin filament branching and connection of the actin cytoskeleton to various transmembrane proteins; scaffold for a wide range of cytoplasmic signaling proteins; tethers cell surface-localized furin, modulates its rate of internalization and directs its intracellular trafficking; defects are the cause of many developmental diseases	5,6, 8,11
Filamin B	MMP-2 MT1-MMP MMP-9	iTRAQ™, ICAT, TAILS ICAT 2DD	ND	cytoplasm, cytoskeleton, sarcomere, Z-disc, cell membrane	Promotion of actin filament branching and connection of the actin cytoskeleton to various transmembrane proteins; scaffold for a wide range of cytoplasmic signaling proteins; defects are the cause of many developmental diseases	5,6, 8,10, 11
Filamin C	MMP-2 MT1-MMP	iTRAQ™, ICAT ICAT	ND	cytoplasm, cytoskeleton, sarcomere, Z-disc, cell membrane	Muscle-specific filamin, putative roles in actin-cross-linking, reorganization of the actin cytoskeleton in response to signaling events, and structural functions at the Z-disks in muscle cells. Critical for normal myogenesis and the structural integrity of the muscle fibers; defects are the cause of myopathy myofibrillar filamin C-related	5,6, 8
Gelsolin	MMP-1,-3 MMP-2 MT1-MMP MMP-9	IVC iTRAQ™, ICAT, TAILS 2D-PAGE 2DD	**(Hwang *et al.*, 2004; Park *et al.*, 2006)**	cytoplasm, cytoskeleton, secreted	F-actin capping and severing; nucleation of F-actin assembly; scavenging of actin and pro-inflammatory components in the plasma; defects are the cause of amyloidosis type 5 or familial amyloidosis Finnish type	1,5, 6,10, 11
IQ motif containing GTPase activating protein 1 (IQGAP1)	MMP-9	2DD	ND	cytoplasm, cytoskeleton, cell membrane, nucleus	Actin crosslinking/bundling; E-cadherin-mediated cell-cell contacts; microtubule capture/polarity; cell motility and invasion; phagocytosis	10
Microtubule-associated protein RP (MAPRE1)	MMP-2 MMP-9	TAILS 2DD	ND	cytoplasm, microtubule network, centrosome	Microtubule formation and stabilization, promoting cell migration; binding and inhibition of the F-actin bundling and microtubule-associated protein APC	10,11
Moesin	MMP-2 MT1-MMP MMP-9	iTRAQ™, ICAT ICAT 2DD, UPLC-MSE	ND	cytoplasm, cell membrane, cell projections, nucleolus, extracellular	Cross-linking of cortical actin filaments and plasma membranes; roles in ECM interactions, cell-cell communication, apoptosis, carcinogenesis and metastasis	5,6, 7,8, 10
Plectin-1	MMP-2 MT1-MMP	TAILS ICAT	ND	cytoplasm, cytoskeleton, cell membrane	Interlinks intermediate filaments with microtubules and microfilaments and anchors intermediate filaments to desmosomes or hemidesmosomes; may bind actin to membrane complexes in muscle	6,8
Profilin-1	MMP-2 MT1-MMP	iTRAQ™, ICAT ICAT	ND	cytoplasm, cytoskeleton, extracellular	G-actin sequestring protein; catalyzes conversion from ADP-actin to ATP-actin; adds actin monomers to the growing end of the actin filament	5,6,8
Stathmin	MMP-9 MMP-1,-2,-8,-13	2DD IVC	**WB**	cytoplasm, cytoskeleton	Regulation of microtubule dynamics by microtubule depolymerization and inhibition of polymerization	10
Trangelin 2	MMP-2 MMP-9	iTRAQ™, TAILS UPLC-MS^{E}	ND	nuclear membrane, cell membrane	Putative actin cross-linking/gelling protein	5,6,7

Tubulin-α/β	MMP-2 MT1-MMP MMP-9 MMP-1,-3,-8,-13	ICAT, TAILS ICAT 2DD IVC	**IVC** ND **IVC** **IVC**	cytoplasm, cytoskeleton, cell projection, extracellular	Heterodimers of α- and β-tubulin chains are the major constituents of microtubules	6,8,10,11
Vimentin	MMP-2 MT1-MMP	iTRAQ™, ICAT, TAILS ICAT	ND	cytoplasm, cytoskeleton, extracellular	Predominant subunit of intermediate filaments found in various non-epithelial cells, especially mesenchymal cells; organizer of critical proteins involved in attachment, migration, and cell signaling	5,6,8,11
PROTEIN BIOSYNTHESIS						
Elongation factor 1-α1	MMP-2 MT1-MMP MMP-9	TAILS ICAT UPLC-MSE, 2DD	ND	cytoplasm, extracellular	Promotion of the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis	7,8,10,11
Elongation factor 1-α2	MMP-2 MT1-MMP	TAILS ICAT	ND	nucleus	Promotion of the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis	8,11
Elongation factor 2	MMP-2 MT1-MMP MMP-9	ICAT, TAILS ICAT UPLC-MSE, 2DD	ND	cytoplasm, extracellular	Promotion of the GTP-dependent translocation of the nascent protein chain from the A-site to the P-site of the ribosome	6,7,8,10,11
Eukaryotic translation initiation factor 1	MMP-2	ICAT, TAILS	ND	cytoplasm	Necessary for scanning and involved in initiation site selection. Promotes the assembly of 48S ribosomal complexes at the authentic initiation codon of a conventional capped mRNA.	6,11
Eukaryotic translation initiation factor 5A	MMP-2 MT1-MMP MMP-9	ICAT ICAT 2DD	ND	cytoplasm, extracellular	Promotion of the formation of the first peptide bond during protein biosynthesis	6,8,10
Alanyl-tRNA synthetase	MMP-2 MT1-MMP	ICAT ICAT	ND	cytoplasm	Catalyzes the attachment of Ala to its cognate tRNA; edits incorrectly charged tRNA(Ala) *via* its editing domain	6,8
Aspartyl-tRNA synthetase	MMP-2	iTRAQ™, TAILS	ND	cytoplasm	Catalyzes the attachment of Asp to its cognate tRNA	5,11
Cysteinyl-tRNA synthetase	MMP-2 MMP-9	iTRAQ™, ICAT 2DD	ND	cytoplasm	Catalyzes the attachment of Cys to its cognate tRNA	5,6,10
Histidyl-tRNA synthetase	MMP-9 MMP-1,-2,-3,-8,-13	2DD, UPLC-MSE IVC	**IVC**	cytoplasm	Catalyzes the attachment of His to its cognate tRNA	7,10
Threonyl-tRNA synthetase	MT1-MMP MMP-9	ICAT 2DD	ND	cytoplasm	Catalyzes the attachment of Thr to its cognate tRNA	8,10
Tryptophanyl-tRNA synthetase	MMP-2	iTRAQ™	ND	cytoplasm	Catalyzes the attachment of Trp to its cognate tRNA; regulation of ERK, Akt, and eNOS activation pathways that are associated with angiogenesis, cytoskeletal reorganization and shear stress-responsive gene expression	5
Valyl-tRNA synthetase	MMP-9	UPLC-MS^{E}	ND	cytoplasm	Catalyzes the attachment of Val to its cognate tRNA	7
REGULATION OF TRANSCRIPTION						
Hepatoma-derived growth factor (HDGF)	MMP-2 MMP-9	iTRAQ™ 2DD	ND	cytoplasm, nucleus, extracellular	Heparin-binding protein with mitogenic activity for fibroblasts; acts as a transcriptional repressor; putative alarmin	6,10
High-mobility group protein B1 (HMGB1)	MMP-2 MT1-MMP MMP-9	iTRAQ™, TAILS ICAT 2DD	ND **WB**	nucleus, cytoplasm, extracellular	Intracellular function: stabilization of nucleosome formation and facilitation of transcription factor binding by bending DNA, role in DNA repair; cytosolic nucleic acid sensor; extracellular function: pro-inflammatory cytokine, alarmin	5,8,10,11
High-mobility group protein B2 (HMGB2)	MMP-2 MT1-MMP MMP-9	iTRAQ™, TAILS ICAT 2DD	ND	nucleus, cytoplasm, extracellular	Intracellular function: stabilization of nucleosome formation and facilitation of transcription factor binding by bending DNA, role in DNA repair; cytosolic nucleic acid sensor	5,8,10,11
High-mobility group protein B3 (HMGB3)	MMP-2	TAILS	ND	nucleus	Intracellular function: stabilization of nucleosome formation and facilitation of transcription factor binding by bending DNA, role in DNA repair; cytosolic nucleic acid sensor; regulation of proliferation and differentiation of common lymphoid and myeloid progenitors	11
Histone H1.2	MMP-2	TAILS	ND	nucleus	Histones H1 are necessary for the condensation of nucleosome chains into higher order structures.	11
Histone H1.3	MMP-2	iTRAQ™	ND	nucleus	Histones H1 are necessary for the condensation of nucleosome chains into higher order structures.	5
Histone H2A	MMP-2	TAILS	ND	nucleus, extracellular	Core component of nucleosome; central role in transcription regulation, DNA repair, DNA replication and chromosomal stability	11

Histone H2B	MMP-2	iTRAQ™	ND	nucleus, extracellular	Core component of nucleosome; central role in transcription regulation, DNA repair, DNA replication and chromosomal stability	5
Histone H4	MMP-2	iTRAQ™	ND	nucleus, extracellular	Core component of nucleosome; central role in transcription regulation, DNA repair, DNA replication and chromosomal stability	5
Lupus La protein	MMP-2 MT1-MMP	ICAT ICAT	ND	nucleus, cell surface	Plays a role in the transcription of RNA polymerase III, most probably as a transcription termination factor as it binds to the 3' termini of virtually all nascent polymerase III transcripts; major autoantigen in SLE	6,8
Nucleobindin-1	MMP-2	iTRAQ™, TAILS	ND	Golgi, cell membrane, extracellular	Ca^{2+}- and DNA-binding protein; B-cell growth and differentiation factor; control of the unfolded protein response; regulation of receptor trafficking	5,11
Nucleolin	MMP-2 MMP-9 MMP-1,-3,-8,-13	iTRAQ™, ICAT 2DD IVC	**WB**	Nucleus, cytoplasm, cell surface	Regulation of RNA polymerase I transcription; folding and maturation of pre-ribosomal RNA; ribosome assembly; nucleo-cytoplasmic transport; histone chaperone activity; interaction with viruses at the cell membrane	5, 6,10
Nucleoside diphosphate kinase B	MMP-2 MMP-9	ICAT 2DD	ND	Cytoplasm, cytoskeleton, nucleus	Major role in the synthesis of nucleoside triphosphates other than ATP; negative regulation of Rho activity; transcriptional activator of the *Myc* gene	6,10
REGULATION OF TRANSLATION						
hnRNP A1	MMP-2 MT1-MMP	TAILS ICAT	ND	cytoplasm, nucleus, spliceosome	Involved in the packaging of pre-mRNA into hnRNP particles, transport of poly(A) mRNA from the nucleus to the cytoplasm and modulatin of splice site selection	8,11
hnRNP A/B	MMP-2	TAILS	ND	cytoplasm, nucleus, ribonucleoprotein complex	Binds ssRNA; high affinity for G-rich and U-rich regions of heterogenous nuclear RNA.	11
hnRNP A2/B1	MMP-2	TAILS	ND	cytoplasm, nucleus, spliceosome	Involved with pre-mRNA processing; forms complexes (ribonucleosomes) with at least 20 other different hnRNP and heterogeneous nuclear RNA in the nucleous.	11
hnRNP A3	MMP-2	TAILS	ND	cytoplasm, nucleus, spliceosome	Plays a role in cytoplasmic trafficking of RNA; binds to the *cis*-acting response element, A2RE; may be involved in pre-mRNA splicing	11
hnRNP D0	MMP-2	ICAT	ND	cytoplasm, nucleus, ribonucleoprotein complex	Binds with high affinity to RNA molecules that contain AU-rich elements (AREs) found within the 3'-UTR of many proto-oncogenes and cytokine mRNAs; binds to ds- and ssDNA sequences in a specific manner and functions a transcription factor; putative roles in telomere elongation and translationally coupled mRNA turnover	6
hnRNP K	MMP-2 MT1-MMP	iTRAQ™, TAILS ICAT	ND	cytoplasm, nucleus, spliceosome	Major pre-mRNA-binding protein; binds tenaciously to poly(C) sequences; probable role in the nuclear metabolism of hnRNAs, particularly for pre-mRNAs that contain cytidine-rich sequences; binds poly(C) single-stranded DNA.	5, 6,8
hnRNP Q	MMP-2 MT1-MMP	ICAT, TAILS ICAT	ND	cytoplasm, microsome, ER, nucleus	Involved with pre-mRNA processing; interacts preferentially with poly(A) and poly(U) RNA sequences; putative roles in translationally coupled mRNA turnover and cytoplasmic vesicle-based mRNA transport through interaction with synaptotagmins	5, 6,8
hnRNP U	MMP-2	TAILS	ND	cytoplasm, nucleus, spliceosome, cell surface	Bind to ds- and ssDNA and RNA; has high affinity for scaffold-attached region (SAR) DNA; inhibits elongation of transcription by polymerase II	11
Nucleophosmin	MMP-2 MMP-9	TAILS UPLC-MS^{E}	ND	nucleus	Involved in diverse cellular processes such as ribosome biogenesis, centrosome duplication, protein chaperoning, histone assembly, cell proliferation, and regulation of tumor suppressors; associated with nucleolar ribonucleoprotein structures and bind ss nucleic acids	7,11
Small nuclear ribonucleo-protein Sm D3	MT1-MMP MMP-9	ICAT 2DD	ND	nucleus, cytoplasm	Component of the spliceosome, involved in the nuclear processing of pre-mRNA	8,10
PROTEIN CHAPERONING						
Calreticulin	MMP-2 MT1-MMP	iTRAQ™, TAILS ICAT	ND	Cytoplasm, ER lumen, nucleus, cell surface, secreted, ECM	Molecular calcium binding chaperone promoting folding, oligomeric assembly and quality control in the ER via the calreticulin/calnexin cycle; extracellular modulation of cell motility and promotion of tumor progression and metastasis	5, 8,11
βB1-crystallin	MMP-9	2D-PAGE	**IVC**	cytoplasm	Prevention of protein unfolding or aggregation; structural component of the eye lens; defects are the cause of cataract	2
Cyclophilin A	MMP-2 MT1-MMP MMP-9 MMP-11	iTRAQ™, ICAT, TAILS ICAT UPLC-MSE, 2DD TAILS	**IVC** ND 	cytoplasm, nucleus, extracellular	Acceleration of protein folding, catalysis of *cis-trans* isomerization of peptidyl-prolyl bonds in oligopeptides; pro-inflammatory functions after secretion	5,6, 7,8, 10,11

Cyclophilin B	MMP-2 MT1-MMP MMP-9	iTRAQ™ ICAT UPLC-MS^{E}	ND	ER lumen, melanosome, extracellular	Acceleration of protein folding, catalysis of cis-trans isomerization of peptidyl-prolyl bonds in oligopeptides	5, 7,8
Cyclophilin D	MMP-2 MT1-MMP	TAILS ICAT	ND	ER lumen, melanosome	Acceleration of protein folding, catalysis of cis-trans isomerization of peptidyl-prolyl bonds in oligopeptides, RNA-binding, putative role in pre-mRNA splicing	8,11
Cyclophilin E	MMP-2 MMP-9	iTRAQ™ 2DD	ND	nucleus	Acceleration of protein folding, catalysis of *cis-trans* isomerization of peptidyl-prolyl bonds in oligopeptides; binding of RNA with potential role in pre-mRNA splicing	5,10
Endoplasmin	MMP-2 MMP-9 MMP-11	TAILS UPLC-MS^{E} TAILS	ND **IVC**	ER lumen, cytoplasm, melanosome	Molecular chaperone that functions in the processing and transport of secreted proteins; functions in ER-associated degradation	6, 7,11
Heat shock protein 27	MMP-9 MMP-11	2DD TAILS	ND	cytoplasm, cytoskeleton, nucleus, cell surface	Molecular chaperone involved in stress resistance and actin organization (actin capping); regulation of metastasis	10,11
Heat shock cognate protein 70	MMP-2 MT1-MMP MMP-9	iTRAQ™, ICAT ICAT UPLC-MSE, 2DD	ND	cytoplasm, cytoskeleton, nucleus, cell surface	Stabilization of pre-existing proteins against aggregation; mediation of the folding of newly translated polypeptides in the cytosol and within organelles	5,6, 7,8, 10
Heat shock protein 70	MMP-2 MT1-MMP	ICAT, TAILS ICAT	ND	cytoplasm, nucleus, extracellular	Molecular chaperone involved in stress resistance; protection of the centrosome during heat shock	6, 8,11
Heat shock protein 75	MMP-2	TAILS	ND	mitochondrion	Molecular chaperone involved in stress resistance; binds to the intracellular domain of tumor necrosis factor type 1 receptor	11
Heat shock protein 90α	MMP-2 MT1-MMP MMP-9 MMP-11	iTRAQ™, TAILS ICAT UPLC-MSE, 2DD TAILS	ND **IVC** ND ND	cytoplasm, cytoskeleton, nucleus, ER lumen, mitochondria, cell surface	Molecular chaperone involved in stress resistance; Role in F-actin bundling and cross-linking; promotion of tumor cell invasion and metastasis	5,6, 7,8, 10,11
Heat shock protein 90β	MMP-2 MT1-MMP MMP-9 MMP-11	TAILS ICAT UPLC-MSE, 2DD TAILS	ND	cytoplasm, melanosome, mitochondrion, extracellular	Molecular chaperone involved in stress resistance	6,7,8, 10,11
Heat shock protein 105	MMP-2	ICAT	ND	cytoplasm, extracellular	Prevents the aggregation of denatured proteins in cells under severe stress, on which the ATP levels decrease markedly. Inhibits heat shock cognate protein 70 ATPase and chaperone activities	6
Oxygen-regulated protein 150	MT1-MMP MMP-9	ICAT 2DD	ND ND	ER lumen, mitochondria	Molecular chaperone, cytoprotective role after oxygen deprivation	8,10
REDOX REGULATION						
Protein DJ-1	MMP-2 MMP-1,-8,-9 MT1-MMP	iTRAQ™, ICAT, TAILS IVC ICAT	**IVC**	cytoplasm, mitochondria, nucleus, extracellular	Redox-reactive signaling intermediate controlling oxidative stress in the brain, protects neurons against oxidative stress and cell death	5,6, 8,11
Peroxiredoxin 1	MMP-2	iTRAQ™, TAILS	ND	cytoplasm, extracellular	Antioxidant by peroxidase activity, control of cytokine-induced peroxide levels which mediate signal transduction	5,11
Peroxiredoxin 2	MMP-2 MMP-9	iTRAQ™ UPLC-MSE, 2DD	ND	cytoplasm, cell membrane	Antioxidant by peroxidase activity, control of cytokine-induced peroxide levels which mediate signal transduction	5, 7,10
Peroxiredoxin 4	MMP-9	2D LC-MS/MS	ND	cytoplasm, secreted	Putative antioxidant by peroxidase acitivity; regulation of the activation of NF-κB in the cytosol by a modulation of IκB-α phosphorylation	9
Peroxiredoxin 5	MMP-2	iTRAQ™	ND	mitochondrion, cytoplasm, peroxisome	Antioxidant by peroxidase activity; involved in intracellular redox signaling	5
Peroxiredoxin 6	MMP-2 MMP-9	iTRAQ™, TAILS 2D-DIGE, 2DD	ND	cytoplasm, lysosome, mitochondrion	Involved in redox regulation of the cell by reduction of H_2O_2 and short chain organic, fatty acid, and phospholipid hydroperoxides; putative roles in the regulation of phospholipid turnover and protection against oxidative injury	4,5, 6,10
SIGNAL TRANSDUCTION						
GDP dissociation inhibitor (GDI) 2	MMP-9	2DD	ND	cytoplasm, cell membrane, extracellular	ND, putative Rab GDP dissociation inhibitor activity	10
Rab GDP dissociation inhibitor (GDI) β	MMP-2 MT1-MMP	ICAT, TAILS ICAT	ND	cytoplasm, cell membrane, cell surface	Regulates the GDP/GTP exchange reaction of most Rab proteins by inhibiting the dissociation of GDP from them, and the subsequent binding of GTP to them	6, 8,11

Rho GDP dissociation inhibitor (GDI) α	MMP-2 MMP-9	TAILS 2DD	ND	cytoplasm, cytoskeleton, extracellular	Regulates the GDP/GTP exchange reaction of the Rho proteins by inhibiting the dissociation of GDP, and the subsequent binding of GTP	6,10
Rho GDP dissociation inhibitor (GDI) β	MMP-2 MMP-9	ICAT 2DD	ND	cytoplasm, cytoskeleton, cell membrane, extracellular	Regulates the GDP/GTP exchange reaction of the Rho proteins by inhibiting the dissociation of GDP, and the subsequent binding of GTP	6,10
14-3-3 protein β/α	MMP-2	TAILS	ND	cytoplasm, melanosome	Adapter protein implicated in the regulation of a large spectrum of signaling pathways by binding and modulating the activity of a large number of partners, usually by recognition of a phospho-Ser or phospho-Thr motif	11
14-3-3 protein η	MMP-2 MT1-MMP	TAILS ICAT	ND	cytoplasm, nucleus, extracellular	Adapter protein implicated in the regulation of a large spectrum of signaling pathways by binding and modulating the activity of a large number of partners, usually by recognition of a phospho-Ser or phospho-Thr motif	8,11
14-3-3 protein γ	MMP-2	iTRAQ™, TAILS	ND	cytoplasm, extracellular	Adapter protein implicated in the regulation of a large spectrum of signaling pathways by binding and modulating the activity of a large number of partners, usually by recognition of a phospho-Ser or phospho-Thr motif	5,11
14-3-3 protein θ	MMP-2 MT1-MMP	iTRAQ™, TAILS ICAT	ND	cytoplasm	Adapter protein implicated in the regulation of a large spectrum of signaling pathways by binding and modulating the activity of a large number of partners, usually by recognition of a phospho-Ser or phospho-Thr motif	5, 8,11
14-3-3 protein ζ/δ	MMP-2 MT1-MMP	TAILS ICAT	ND	cytoplasm, mitochondrion, nucleus, extracellular	Adapter protein implicated in the regulation of a large spectrum of signaling pathways by binding and modulating the activity of a large number of partners, usually by recognition of a phospho-Ser or phospho-Thr motif	8,11
APOPTOSIS						
Apoptosis-linked-gene-2-interacting-protein X (Alix)	MMP-9	2DD	ND	cytoplasm, melanosome, centrosome, extracellular	Control of the production of and trafficking through endosomes called multivesicular bodies; regulation of caspase-dependent and caspase-independent cell death	10
BH3 interacting domain death agonist (BID)	MMP-9	2DD	ND	cytoplasm, mitochondrial membrane	Major proteolytic product p15 BID: release of cytochrome c; induction of ICE-like proteases and apoptosis; countering the protective effect of Bcl-2	10
Calpain 2	MMP-2 MT1-MMP	ICAT ICAT	ND ND	cytoplasm, cell membrane	Ca^{2+}-regulated non-lysosomal thiol-protease which cleaves substrates involved in cytoskeletal remodeling, apoptosis and signal transduction.	6,8
Calpastatin	MT1-MMP	ICAT	ND	cytoplasm	Specific inhibitor of calpains	8
Cytochrome c	MMP-11	TAILS	ND	mitochondrial matrix, cytosol, nucleus	Transfer of electrons to the cytochrome oxidase complex, the final protein carrier in the mitochondrial electron-transport chain; released in cytosol during apoptosis resulting in activation of caspase-9	11
LYSOSOMAL DEGRADATION						
β-glucuronidase	MMP-9	2D LC-MS/MS, 2DD	ND	lysosome	Important role in lysosomal degradation of dermatan and keratan sulfates; defects are the cause of mucopolysaccharidosis type 7 or Sly syndrome	9,10
Cathepsin A (lysosomal carboxypeptidase A)	MMP-2	ICAT	ND	lysosome, ER, extracellular	Protective protein appears to be essential for both the activity of β-galactosidase and neuraminidase, it associates with these enzymes and exerts a protective function necessary for their stability and activity.	6
Cathepsin B	MMP-2 MT1-MMP	iTRAQ™, TAILS ICAT	ND	lysosome, mitochondrion, melanosome	Thiol protease which is believed to participate in intracellular degradation and turnover of proteins. Has also been implicated in tumor invasion and metastasis.	5, 8,11
Cathepsin D	MMP-9	UPLC-MS^E	ND	lysosome, melanosome, extracellular	Acid protease active in intracellular protein breakdown. Involved in the pathogenesis of several diseases such as breast cancer and possibly Alzheimer disease; defects are the cause of neuronal ceroid lipofuscinosis type 10	7
Cathepsin E	MMP-9	2D LC-MS/MS	ND	endosome, ER, Golgi	May have a role in immune function. Probably involved in the processing of antigenic peptides during MHC class II-mediated antigen presentation. May play a role in activation-induced lymphocyte depletion in the thymus, and in neuronal degeneration and glial cell activation in the brain.	9
Cathepsin L1	MMP-2 MT1-MMP	iTRAQ™, ICAT, TAILS ICAT	ND	lysosome, extracellular	Important for the overall degradation of proteins in lysosomes.	5,6, 8,11
Cathepsin Z (or X)	MMP-9	UPLC-MS^E	ND	lysosome, ER	Implicated in the promotion of cancer progression	7

Iduronate-2-sulfatase (IDS)	MMP-2,-8,-9 MT1-MMP	ICAT	**IVC**	lysosome	Required for the lysosomal degradation of heparan sulfate and dermatan sulfate; defects are the cause of mucopolysaccharidosis type 2 or Hunter disease	8
Niemann-Pick, type C2 (NPC2)	MT1-MMP	ICAT	**IVC**	lysosome, secreted	Key role in cellular cholesterol homeostasis by participating in intracellular cholesterol trafficking and the production production of low-density lipoprotein cholesterol-derived oxysterols; defects cause the lysosomal storage disease Niemann-Pick disease type C2	8
UBIQUITINATION						
Ubiquitin-activating enzyme E1	MMP-2	TAILS	ND	cytoplasm, nucleus	Activates ubiquitin by first adenylating its C-terminal glycine residue with ATP, and thereafter linking this residue to the side chain of a cysteine residue in E1, yielding an ubiquitin-E1 thioester and free AMP.	11
Ubiquitin-conjugating enzyme E2 variant 1	MMP-9	2DD	ND	cytoplasm, nucleus	Catalysis of non-canonical poly-ubiquitin chain synthesis; role in the activation of NF-κB; transcriptional activation of target genes; role in the control of cell cycle and differentiation; role in error-free DNA repair pathway; contribution to cell survival after DNA damage	1
Ubiquitin-conjugating enzyme E2 L3	MMP-2	ICAT	ND	cytoplasm, nucleus	Catalyzes the covalent attachment of ubiquitin to other proteins, mediating the selective degradation of short-lived and abnormal proteins	6
Ubiquitin-conjugating enzyme E2N-like	MMP-9	2DD	ND	nucleus	ND, belongs to the ubiquitin-conjugating enzyme family	10
MISCELLANEOUS						
Annexin I	MMP-2 MMP-9	iTRAQ™ 2DD	ND **WB**	cytoplasm, cell projection cell membrane, nucleus, extracellular	Calcium/phospholipid-binding protein which promotes membrane fusion and is involved in exocytosis; regulation of phospholipase A2 activity;inhibition of neutrophil extravasation and induction of neutrophil apoptosis	5,10
Carbonic anhydrase II	MMP-9 MMP-13	2DD IVC	**NIVC** **IVC**	cytoplasm, nucleus, extracellular	Reversible hydration of carbon dioxide; essential for bone resorption and osteoclast differentiation; defects are the cause of renal tubular acidosis or Guibaud-Vainsel syndrome	10
Ferritin light chain	MMP-2 MMP-9	iTRAQ™ 2DD	ND	cytoplasm, extracellular	Storage of iron in a soluble, non-toxic, readily available form; defects are the cause of hereditary hyperferritinemia-cataract syndrome and neuroferritinopathy	5,10
Galectin-1	MMP-2 MT1-MMP MMP-9 MMP-11	iTRAQ™, TAILS ICAT UPLC-MS^{E} TAILS	ND **IVC** ND **IVC**	cytoplasm, ECM	May regulate apoptosis, cell proliferation and cell differentiation. Binds a wide array of complex carbohydrates. Affects T cell homeostatis by inhibiting CD45 protein phosphatase activity; putative alarmin	5,7, 8,11
Galectin-3	MMP-2,-9	iTRAQ™	**(Ochieng *et al.*, 1994; Ochieng *et al.*, 1998)**	nucleus, cytoplasm, mitochondrion, cell membrane, secreted, cell surface	Galactose-specificlectin which binds IgE.; may mediate integrin-dependent stimulation of endothelial cells migration.	5
Myosin light chain	MMP-2	2D-PAGE	**IVC**	cytoplasm, cytoskeleton, sarcomere, extracellular	Mechanical protein that generates force during muscle contraction	3
Progranulin	MT1-MMP	ICAT	**IVC**	cytoplasm, extracellular	Pluripotent growth factor with roles in development, wound repair, inflammation and tumorigenesis; defects cause ubiquitin-positive frontotemporal dementia	8

ADP, adenosine diphosphate**; APC,** adenomatous polyposis coli; **AMP,** adenosine monophosphate; **ATP,** adenosine triphosphate; **CMP**, cytidine monophosphate; **dsDNA**, double-stranded DNA; **ECM,** extracellular matrix; **E-cadherin,** epithelial cadherin; **ER,** endoplasmic reticulum; **F-actin,** filamentous actin; **G-actin,** globular actin; **hnRNP,** heterogenous nuclear ribonucleoprotein; **ICE**, interleukin-1β-converting enzyme; **IgE**, immunoglobulin E; **IκB-α,** inhibitor of NFκB-α; **IVC**, *in vitro* cleavage; **ND,** not defined; **NFκB**, nuclear factor κB**; ssDNA,** single-stranded DNA; **UMP,** uridine monophosphate; **SLE,** systemic lupus erythematosus.

[*]Addition of a substrate to the list of high confidence candidate substrates was based on the identification in various degradomics approaches, the (putative) cleavage by more than one MMP, the identification of a protein of the same subfamiliy or with a similar function as an MMP (candidate) substrate or the biochemical confirmation by *in vitro* cleavage (IVC) or Western blot analysis (WB).

[†]Degradomics method used for the identification of the (candidate) substrate. The meaning of the acronyms as well as explanations on the methods can be found in Table 1.

[‡]Confirmation; candidate substrates may be confirmed biochemically by cleavage of the recombinant/purified substrate *in vitro* (**IVC**), or by Western blot analysis (**WB**) of digested cytoplasm with substrate-specific primary antibodies. **NIVC**, not cleaved *in vitro*.

[¶]References are coded as follows: 1,(Hwang *et al.*, 2004); 2, (Descamps *et al.*, 2005); 3,(Sawicki *et al.*, 2005); 4, (Greenlee *et al.*, 2006) ; 5,(Dean and Overall, 2007); 6, (Dean *et al.*, 2007); 7,(Xu *et al.*, 2008); 8,(Butler *et al.*, 2008); 9,(Vaisar *et al.*, 2009); 10,(Cauwe *et al.*, 2009); 11,(Kleifeld *et al.*, 2010).

3.1 Intracellular MMP detection: methodological considerations

Classical SDS-PAGE-based gelatin zymographic analysis, henceforth called 'substrate zymography', to detect activated forms of MMP-9 and MMP-2 does not reveal the actual *in situ* activity of the proteases. During SDS-PAGE analysis, MMPs undergo an artificial nonproteolytic activation by the detergent SDS, which denatures the proteases and deviates the propeptide out of the catalytic side (*vide supra*). After the SDS-PAGE, SDS is washed away using Triton X-100 and the proteases refold, but the propeptide remains detached from the catalytic site, resulting in gelatin degradation by artefactually active pro-MMP-9 and pro-MMP-2. In contrast, nitrosative-stress induced activation of MMPs results in a size modification that is too small to be detected by SDS-PAGE (*cf.* 1.2). In the latter case, the pro-MMP may be a biologically active enzyme. In addition, SDS separates the MMPs from their natural inhibitors, i.e. the TIMPs. As a consequence, in the presence of TIMP-1/MMP-9 or TIMP-2/MMP-2 ratios in excess of 1, substrate zymographic analysis erroneously suggests MMP activities although in the biological samples or eventually in the *in situ* situation, they may be kept inactive by TIMP binding. In addition, one needs to keep in mind that not all fragments of pro-MMPs are active, because intermediate activation forms exist and that *ex vivo* active forms may be under endogenous inhibition *in vivo*.

Detection of MMPs by substrate zymography of cell lysates excludes determination of subcellular localization and may result in artefactual activation of the enzymes by detergents in the cell lysis buffer. Detergent-soluble fractions of cellular lysates also contain membrane-associated proteins and as a consequence do not reflect intracellular localization *per se*. Hence, native i.e. non-denaturing subcellular fractionation into membrane, cytosolic, mitochondrial, nuclear and other organelle fractions, prior to the addition of detergents, is a prerequisite to demonstrate intracellular presence of activated MMPs by substrate zymography. Ideally, fractionation efficiency needs to be checked by assessing the distinctive presence of fraction-specific proteins, e.g. histone H3, actin and ICAM-1 as markers for nuclear, cytoskeletal and membrane fractions, respectively. Co-immunoprecipitation of the MMP and its substrate from the specific cell fraction gives an additional confirmation of subcellular colocalization of target and protease. To gain information on actual subcellular activity, the gel-based zymographic assay was adapted to assess net proteolytic activity *in situ* (Galis *et al.*, 1995). A substrate, e.g. gelatin or a peptide, is fluorescently labeled and applied to tissue sections. *In situ* zymography preserves the fine morphological details of the tissue or cell monolayer. However, whereas MMP-2 and MMP-9 are the most active gelatin degraders (Mackay *et al.*, 1990), gelatin is not an exclusive substrate of the gelatinases, but is also digested by other MMPs such as MMP-1, MMP-3, MMP-7, MMP-13/collagenase-3 and MT-MMPs (Sang and Douglas, 1996; Pavlaki and Zucker, 2003). Possible candidates able to digest both gelatin and type IV collagen are MMP-3 and MMP-7 (Sang and Douglas, 1996), MMP-26 (Park *et al.*, 2000) and serine proteases such as cathepsins (Bailey, 2000). A critical reader will take into consideration that the key messages here are the relative amounts of the various substrate-cleaving enzymes and their specific activities in substrate conversions. For example, a protease with a 100-fold lower activity on gelatin compared with for example, MMP-9, will need a 100-fold higher concentration to be detected by *in situ* zymography with the same intensity as MMP-9. Likewise, activity assays based on fluorigenic peptides are rarely specific and must be combined with the use of specific neutralizing antibodies or cells of wildtype and MMP knockout mice.

Many proteases act in cascades (also called 'the protease web' or the 'proteolytic internet', *cf.* section 1.1). Hence, proteases (and inhibitors) may be the substrates of other MMPs. As a consequence, it will often be difficult to define a direct cleavage, because the substrate conversion may be indirect, i.e. caused by an upstream protease. Such situations may be resolved *in vitro* (e.g. with the use of ultrapure substrates and proteases). However, to discriminate between direct and indirect substrates *in vivo*, highly specific inhibitors need to be developed first.

At present, the only safe approach to establish a causal relationship between a protease and cleavage of a specific substrate *in vivo* is by comparing wildtype and mice rendered genetically deficient in the particular protease. Of course, this approach has drawbacks and limitations i.e. the knockout mice might not be viable or subfertile, pushing an already tedious procedure to its technical limits. The comparisons need to be done in backcrossed animals and, to avoid genetic confounding, these backcrosses need to be performed ideally for more than 13 generations. In addition, one needs to keep in mind that differences, genetic and compensatory ones, will always exist between men and mice (Hu *et al.*, 2007). The question remains whether any of the established MMP knockout mice provide credence for intracellular MMP activities. The answer to this question is not simple. First, spontaneous phenotypes in MMP knockouts are limited and, to our knowledge, no intracellular substrates have been detected so far in healthy animals, except for pro-cryptdins (Wilson *et al.*, 1999). Second, in induced phenotypes of infection and inflammation, various intracellular substrates have been defined *in vivo*, e.g. βB1-crystallin (Descamps *et al.*, 2005), connexin-43 (Lindsey *et al.*, 2006), zona occludens-1 (Asahi *et al.*, 2001) and X-ray cross-complementary factor 1 (Yang *et al.*, 2010). It may be, however, that in some of these substrates, the cleavage occurs extracellularly.

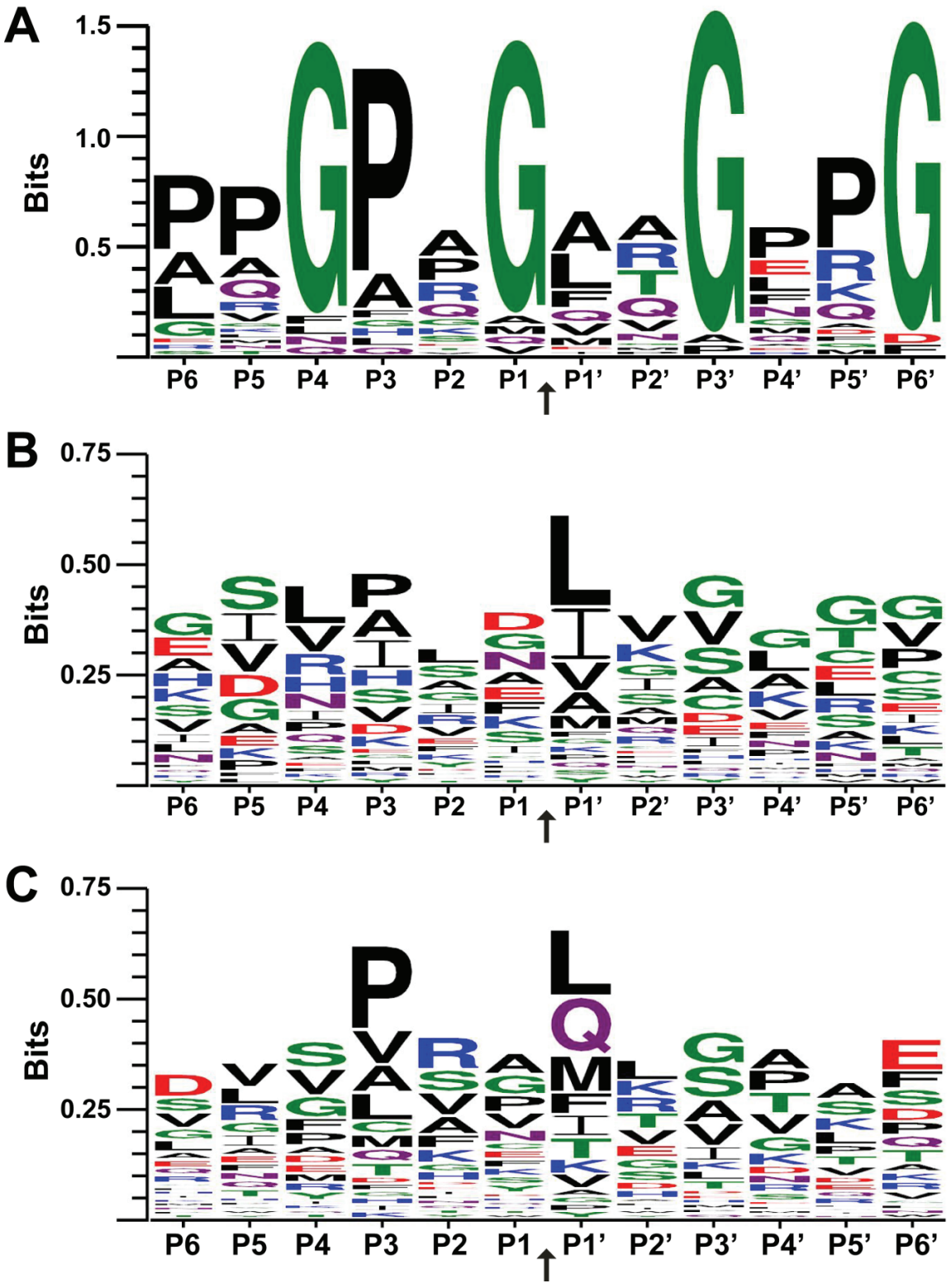

Figure 4. Comparison of the MMP-9-mediated cleavage sites in collagen and gelatin, in extracellular substrates and in intracellular substrates. Cleavage site specificities are visualized by sequence logos, created with the bio-informatics software WebLogo 3 and with the application of small sample adjustments and corrections for natural amino acid abundances (Crooks *et al.*, 2004; Schneider and Stephens, 1990). The overall height of the stack is a measure of sequence consensus, while the heights of the amino acid symbols within each stack indicate the relative frequency of each amino acid in that position. MMP-9 cleaves the peptide bonds between positions P1 and P1' (arrow). **A.** Cleavage preferences in gelatin type II, procollagen type II and collagen type V (Van den Steen *et al.*, 2002). **B.** Cleavage specificities in extracellular substrates (secreted and membrane-associated) (Cauwe *et al.*, 2007; Van den Steen *et al.*, 2002; Lin *et al.*, 2008; Chow *et al.*, 2008; Xu *et al.*, 2008; Vaisar *et al.*, 2009). **C.** Intracellular substrate specificities (Cauwe *et al.*, 2009; Descamps *et al.*, 2005; Starckx *et al.*, 2003; Levin *et al.*, 2009). Although differences exist between the three sequence logos, a general preference subsists in all substrates for hydrophobic amino acids at P1', small amino acids at P1 (Gly, Ala) and a Pro residue at P3, as previously described (Van den Steen *et al.*, 2002).

An alternative approach is the use of specific protease inhibitors. However, small-molecule inhibitors inhibiting only a single MMP have not been discovered so far and the only way of specific inhibition is the use of highly specific neutralizing antibodies (Hu *et al.*, 2007). A third associative method may also be used with patient samples. Indeed, significant inverse correlations between intact substrate and MMP levels in large groups of biological (patient) samples are strongly suggestive of *in vivo* cleavage. However, it is important to keep in mind that such correlations may be indicative of other associations than pure causal relationships.

As will be outlined, many substrates are cleaved by MMPs. It remains a methodological challenge to verify and validate whether such substrates are biologically relevant. With the use of unbiased methods to define new substrates, it is possible that biologically irrelevant substrates are detected. However, it is equally possible that by such approaches unanticipated relevant new functions of MMPs and substrates are discovered in physiology and pathology.

Substrate cleavage sites *in vitro* may be safely determined in two ways. After cleavage of the substrate in solution, the intact molecule and the fragments are separated by high performance liquid chromatography (HPLC). Mass spectrometry (MS) analysis of the elution fractions determines the mass and thus the sequence of the peptide fragments, as compared with database searches of theoretical peptide masses (Lopez-Otin and Overall, 2002). Alternatively, the fragments may be separated by SDS-PAGE analysis and electroblotted onto PVDF for identification of the fragments NH_2-termini by Edman degradation (Edman, 1970). Excision of fragment bands out of SDS-PAGE gels and subsequent MS analysis after in-gel trypsin digests will not yield cleavage sites and may only be indicative of cleavage regions. Indeed, unless the method is extremely well calibrated and sensitive, not all peptides may be captured and this will result in false positives. It is always advised to use complementary techniques to corroborate identified *in vitro* cleavage sites. In addition, with presently available tools for high-throughput substrate and cleavage site identification (*cf.* Table 1), it will be even more challenging to demonstrate that such cleavages are biologically meaningful. Since MMPs do not cleave at stringent substrate recognition sites, but only possess general preferences, *in silico* predicted cleavage sites are theoretical and need to be confirmed by one of the above methods or by generation and analysis of substrates with mutated cleavage sites.

3.2 Subcellular localization mechanisms of MMPs

As discussed in chapter 1, MMPs may be activated in the extracellular milieu or inside cells. In this chapter we will summarize the present data on localization of MMPs in various subcellular compartments and the concomitant pathophysiological consequences. In addition, we will discuss the information about the mechanisms used by specific MMPs to enter cells and to translocate to different intracellular locales. These data are summarized in Table 4, showing for each MMP the various known subcellular localizations and translocation mechanisms in specific cell types, the (putative) intracellular activation modes, the intracellular substrates and the pathophysiological effects of intracellular translocation.

3.2.1 Cytosolic MMP activity

The cytoplasm refers to the portion of the cell that is enclosed by the plasma membrane and is a collective term for the cytosol plus the organelles suspended in the cytosol. When intracellular localization of MMPs was determined solely on the basis of immunostainings, and not with additional confirmation by subcellular fractionation (*cf.* section 3.1), discrimination between cytosolic and/or organelle staining is difficult. Hence, in these cases we use the more general term, cytoplasm. In addition, in the cytoplasm we only consider activated MMPs, since presence of pro-MMPs in the cytoplasm is mostly associated with protein synthesis in the endoplasmatic reticulum (ER) and the Golgi apparatus, and reflects MMPs that are prepared for secretion (*vide infra*).

Many proteins have inefficient signal sequences, which may result in a variable fraction (5-20%) that is not translocated across the ER membrane but instead remains in the cytosol (Hegde and Bernstein, 2006). An example of this mechanism is the ER chaperone protein calreticulin, which has additional functions in the cytosol and nucleus (Shaffer *et al.*, 2005). Likewise, the signal sequence at the NH_2-terminus of MMP-3 contains a Pro residue at the fifth position, which was proposed to work as a helix breaker, and results in an inefficient signal peptide and sorting of MMP-3 to both the cytoplasm and the ER-Golgi pathway for further secretion (Eguchi *et al.*, 2008). Since a Pro at the start of the signal sequence is found in many MMPs, for example MMP-1,-8,-9,-13 and MT1-MMP, it would be interesting to investigate whether signal sequence inefficiency may be a general mechanism for dual sorting of MMPs to the cytosol and to the extracellular space.

Conversely, some MMPs have been found to enter cells by endocytosis. Endocytotic uptake occurs by various mechanisms, which can be roughly divided into those that are clathrin-dependent and those that are clathrin-independent. In clathrin-dependent endocytosis, the cytoplasmic domains of membrane-associated receptors or 'cargo proteins' are specifically recognized by adaptor proteins and packaged into clathrin-coated vesicles that are brought into the cell (Traub, 2009; Grant and Donaldson, 2009). Clathrin-independent endocytosis is less well studied and encompasses many different endocytic mechanisms, such as caveolae- and flotilin-dependent endocytosis, uptake in GPI-enriched early endosomal compartments (GEEC) and actin-driven

pathways such as macropinocytosis and phagocytosis (Grant and Donaldson, 2009; Doherty and McMahon, 2009).

MMP-7 may be internalized by clathrin-dependent endocytosis, since two inhibitors of clathrin-dependent endocytosis (phenylarsine oxide and chlorpromazine) inhibit the ability of exogenously added MMP-7 to cleave the intraneuronal protein synaptosomal-associated protein of 25 kDa (SNAP-25) (Szklarczyk *et al.*, 2007b). In support of this, immunostaining for MMP-7 showed translocation of exogenously added MMP-7 to the cytoplasmic compartment. Exogenous MMP-3 was shown to enter the cytoplasm of chondrosarcoma cells and to further translocate to the nucleus (Eguchi *et al.*, 2008). Laser confocal microscopy analysis showed colocalization of MMP-3 with low-density lipoprotein-related protein 1 (LRP1), suggesting that MMP-3 may enter cells by clathrin-dependent endocytosis (Traub, 2009). Other MMPs are also internalized by LRP-mediated endocytosis, including MMP-2 (Emonard *et al.*, 2004; Yang *et al.*, 2001), MMP-9 (Hahn-Dantona *et al.*, 2001; Van den Steen *et al.*, 2006) and MMP-13 (Barmina *et al.*, 1999; Raggatt *et al.*, 2006).

Regardless of the mode of entry, endocytosed cargo proteins and their loads are usually delivered to the early endosome. The lumen of the early endosome is mildly acidic, which facilitates ligand release from the cargo receptors. The cargo proteins can be routed to the late endosomes and lysosomes for degradation, to the *trans*-Golgi network (TGN), or to recycling endosomal carriers that recycle the cargo proteins back to the plasma membrane (Grant and Donaldson, 2009). However, the internalized ligand proteins remain in the endosomal lumen and will be transported to lysosomes for degradation. Indeed, LRP-mediated endocytosis was described as a clearance mechanism to eliminate excessive MMP activity (Emonard *et al.*, 2005) and it is not clear how MMPs may escape endosomal entrapment and enter the cytosol. Some bacterial toxins and viruses are able to leave the endosomes by pH-dependent conformational changes (Cho *et al.*, 2003) or by pH-independent mechanisms (Matsuzawa *et al.*, 2004). Inhibition of endosomal acidification does not prevent SNAP-25 cleavage by exogenous MMP-7. Hence, MMP-7 uses an undetermined pH-independent mechanism to escape endosomal entrapment.

MT1-MMP is internalized by both clathrin-dependent (Jiang *et al.*, 2001a; Uekita *et al.*, 2001) and clathrin-independent caveolae-dependent mechanisms (Remacle *et al.*, 2003; Galvez *et al.*, 2004) and is recycled to the cell surface, which may be a mechanism to relocalize active MT1-MMP at the leading edge during cell migration (*cf.* 3.3.2). Caveolae are small membrane invaginations formed by the polymerization of caveolins and contain a subset of lipid-raft components, such as sphingolipids and cholesterol. Caveolae bud off the plasma membrane to form endocytic caveolar carriers that fuse with the caveosome or with the early endosome, or can fuse back to the plasma membrane (Parton and Simons, 2007). MMP-2 was found to colocalize with MT1-MMP, TIMP-2 and caveolin on the surface of endothelial cells (Puyraimond *et al.*, 2001) and cardiomyocytes (Chow *et al.*, 2007a). Again, the question is raised about the mechanisms that MT1-MMP and MMP-2 may use to access their cytoplasmic targets (*vide supra*). Indeed, after endocytosis, the cytoplasmic tail of MT1-MMP faces the cytoplasm, whereas the catalytical domain resides in the endosomal lumen. Hence, it would be interesting to investigate whether MT1-MMP could flip-flop, i.e. flip its orientation from inwards (as it is after endocytosis) to outwards (with its catalytic site in the cytosol and its tail in the endosome). Mechanisms for lipid flip-flop in cellular membranes have been described and are important in various biological processes such as membrane biogenesis, vesicle formation and the biosynthesis of glycolipid precursors of cell surface glycoconjugates (Pomorski and Menon, 2006). The same mechanism may apply for MMP-2 as it associates with the endocytic membrane through the binding of the caveolin scaffolding domain of caveolin-1 and caveolin-3 (Chow *et al.*, 2007a) or by association with TIMP-2 and MT1-MMP (Puyraimond *et al.*, 2001).

3.2.2 MMP activity in the secretory pathway

Classically, vesicles carrying secretory proteins are transported from the endoplasmatic reticulum (ER) to the Golgi and subsequently to the TGN. As discussed in section 1.1, MMP-11 and MT3-MMP are activated by furin within the TGN (Kang *et al.*, 2002; Pei and Weiss, 1995; Santavicca *et al.*, 1996). Likewise, treatment of vaginal-cervical epithelial cells with estrogen stimulates intracellular activation of MMP-7 in the Golgi apparatus (Gorodeski, 2007). Furthermore, resting neutrophils contain pro- and activated forms of MT6-MMP, which is distributed among specific granules, gelatinase B granules, secretory vesicles and the plasma membrane (Kang *et al.*, 2001). It is at present unknown whether MMP-8/neutrophil collagenase and MMP-9/gelatinase B may be activated intracellularly by MT6-MMP in neutrophils. However, the classical view is that these enzymes are rapidly activated after neutrophils degranulation, e.g. by HOCl (Peppin and Weiss, 1986; Weiss *et al.*, 1985) (*cf.* section 1.2). Upon treatment with phorbol 12-myristate 13-acetate (PMA), microvascular endothelial cells accumulate activated forms of MMP-9. In addition, both pro- and activated forms of MMP-2 were found in secretory vesicles of neuroblastoma and primary neurons and reactive astrocytes (Sbai *et al.*, 2010; Sbai *et al.*, 2008). In reactive astrocytes and melanoma cells, MMP-2 and MMP-9 were found in different vesicle populations aligned along microfilaments and/or microtubules (Sbai *et al.*, 2010; Schnaeker *et al.*, 2004). In the context of the dogma of extracellular action of MMPs, the presence of intracellularly activated MMPs in the secretory pathway and in secretory vesicles was considered as a means for

rapid secretion of MMP activity in the extracellular milieu. However, some MMP-2- and MMP-9-containing vesicles in neurons and melanoma cells also undergo retrograde transport, suggesting that these vesicles are not intended for secretion (Schnaeker *et al.*, 2004; Sbai *et al.*, 2008). In addition, since communication exists between the Golgi network and the endosomal pathway for the recycling of cargo proteins (Grant and Donaldson, 2009), it would be interesting to investigate whether proteins within the Golgi apparatus may be exchanged and taken up into the endosomal pathway, from where they may enter the cytosol by previously suggested mechanisms (*cf.* 3.2.1). Alternatively, flip-flop mechanisms may orient vesicular MT-MMPs to the cytosolic space, as was suggested for endosomal escape.

3.2.3 MMP activity associated with the cytoskeleton

The intracellular matrix (ICM) consists of the actin cytoskeleton and the microtubular network. The ICM preserves the cellular shape, enables cell migration and intracellular transport and plays major roles in cell division. MMP-2 cleaves the cytoskeletal proteins desmin and α-actinin and colocalizes with α-actinin in cardiomyocytes (Sung *et al.*, 2007). As previously mentioned, MMP-2- and MMP-9-containing vesicles are aligned with the cytoskeleton in neurons and reactive astrocytes, and both gelatinases are found in cytoskeletal fractions from these cells (Sbai *et al.*, 2010; Sbai *et al.*, 2008). Disruption of the actin cytoskeleton with cytochalasin D in astrocytes results in the partial recruitment of MMP-2 and gelatinolytic acitivity into actin aggregates, which further indicates a connection between gelatinases and the actin cytoskeleton (Ogier *et al.*, 2006). Both MT1-MMP and MT3-MMP are detected in cytoskeletal fractions of smooth muscle cells, where they cleave the cytoskeletal protein focal adhesion kinase (FAK) (*vide infra*) (Shofuda *et al.*, 2004). In addition, MT1-MMP is trafficked along the microtubule to the centrosomal compartment, where it induces malignant transformation by cleaving the integral centrosomal protein pericentrin (*vide infra*) (Golubkov *et al.*, 2006; Golubkov *et al.*, 2005b; Golubkov *et al.*, 2005a). Moreover, cytoskeletal proteins constitute an important fraction of the intracellular degradomes of MMP-2, MMP-9 and MT1-MMP, as determined by various degradomics methods (*cf.* Table 3). Hence, the functional analysis of the effect(s) of ICM cleavage by MMPs may lead to novel insights, some of which will be suggested throughout the manuscript.

3.2.4 MMP activity in the sarcomere

The cardiac myocyte is a specialized striated muscle cell that is composed of bundles of myofibrils that contain myofilaments. The myofibrils have distinct, repeating units called sarcomeres, which represent the basic contractile units of the myocyte. MMP-2 was localized to the sarcomere of cardiac myocytes by various methods including immunogold electron microscopy, confocal microscopy, substrate zymography analysis of highly purified thin myofilament preparations and coimmunoprecipitation with sarcomere proteins that are cleaved by MMP-2 during cardiac injury, causing myocardial dysfunction (*vide infra*) (Gao *et al.*, 2003; Kandasamy *et al.*, 2010; Sawicki *et al.*, 2005; Wang *et al.*, 2002a). In addition, in dilated cardiomyopathy, both MMP-2 and MMP-9 are localized to the sarcomere, where they cleave myosin heavy chain (Rouet-Benzineb *et al.*, 1999). Under physiological circumstances, these MMP activities may be kept in check by sarcomeric TIMP-4 (Schulze *et al.*, 2003).

3.2.5 Mitochondrial MMP activity

Mitochondria may be considered as the power plants of the cell and are crucial for numerous cellular processes, including apoptosis, signaling, and metabolic pathways involving lipids, amino acids and iron. Both pro- and activated MMP-1 are associated with the mitochondrial membrane in glial Müller cells, Tenon's capsule fibroblasts, corneal fibroblasts and retinal pigment epithelial cells (Limb *et al.*, 2005). Since the mitochondrial localization of MMP-1 is found in resting cells, it suggests a physiological role for MMP-1 in cellular homeostasis. In addition, mitochondrial MMP-1 confers resistance to apoptosis by uncharacterized mechanisms. Both MMP-2 (Wang *et al.*, 2002b; Kwan *et al.*, 2004) and MMP-9 (Moshal *et al.*, 2008) are detected in cardiac mitochondria during cardiac injury and increased levels of mitochondrial MMP-9 are associated with exacerbated mechanical dysfunction.

3.2.6 MMP activity in the nucleus

Surprisingly, many studies report nuclear localization of MMPs, including MMP-1,-2,-3,-9,-13,-26 and MT1-MMP (*cf.* Table 4), and cleavage of nuclear matrix proteins (*cf.* Tables 3 and 5). The nuclear matrix binds more than 200 nuclear proteins and supports their assembly into functional macromolecular complexes involved in important nuclear processes, such as transcription, RNA splicing and DNA replication (Zink *et al.*, 2004). The nuclear matrix is surrounded by a nuclear lamina and a double-membrane nuclear envelope. Hence, nuclear import and export mechanisms are needed for the movement of large macromolecules into and out of the nucleus (Quimby and Corbett, 2001). As a consequence, to enter or exit the nucleus, MMPs need internal signals, termed nuclear localization signals (NLSs) or nuclear export signals (NESs). These signals are recognized by soluble receptors that mediate macromolecular transport through the nuclear pore complex. Two types of human NLSs exist, i.e. classical basic Lys-rich and M9-type, which are recognized by importins/karyopherins-α and transportin (importin/karyopherin-β2), respectively. Classical NESs are characterized by the presence of Leu residues and are recognized by the exportin CRM1 (Benmerah *et al.*, 2003; Quimby and Corbett, 2001).

Table 4. Intracellular localization of MMPs and TIMPs

MMP	Subcellular localization	Cell type	Localization mechanism	Activation mode	Substrates*	Pathophysiological effect	Reference
MMP-1	**mitochondria, nucleus**	glial Müller cells, Tenon's fibroblasts, RPE cells	ND	ND	ND	Resistance to apoptosis	(Limb *et al.*, 2005)
MMP-2	**caveolae**	endothelial cells, cardiomyocytes	CAV-endocytosis	z	ND	ND, putative regualtion of intracellular localization and function of MMP-2	(Puyraimond *et al.*, 2001; Chow *et al.*, 2007a)
	sarcomere	cardiomyocytes	ND	oxidative stress	TnI, MLC-1, MHC	Contributes to mechanical dysfunction of the heart after ischemia-reperfusion injury, cytokine-induced myocardial dysfunction and in dilated cardiomyopathy	(Rouet-Benzineb *et al.*, 1999; Gao *et al.*, 2003; Sawicki *et al.*, 2005; Wang *et al.*, 2002b)
	cytoskeleton	cardiomyocytes	ND	oxidative stress (putative)	α-actinin, desmin	ND, may contribute to cardiac dysfunction induced by peroxynitrite	(Sung *et al.*, 2007)
	cytoplasmic vesicles, cytoskeleton, nucleus	astrocytes	ND	ND	ND	ND	(Sbai *et al.*, 2010)
	secretory vesicles	hippocampal neurons	secretion pathway	ND	ND	ND	(Sbai *et al.*, 2008)
	mitochondria	cardiomyocytes	ND	ND	ND	ND	(Kwan *et al.*, 2004; Wang *et al.*, 2002a)
	cytosol	cardiomyoblasts	ND	oxidative stress	GSK-3β	Augmentation of GSK-3β kinase activity, which may contribute to cardiac injury resulting from enhanced oxidative stress	(Kandasamy and Schulz, 2009)
	cytosol, nucleus	endothelial cells	ND	oxidative stress (putative)	ND	ND, putative function in the degradation of nuclear matrix in cigarette smoke-induced apoptosis	(Ruta *et al.*, 2009)
	nucleus	cardiomyocytes, hepatocytes	NLS	oxidative stress (putative)	PARP-1	ND, putative removal of excess PARP-1 during oxidative stress, sparing the cell from ATP depletion	(Kwan *et al.*, 2004)
	nucleus	neurons	ND	MT1-MMP (putative)	PARP-1, XRCC1	Contribution to oxidative DNA damage in neurons early after ischemic insult, protective effect in later stages	(Yang *et al.*, 2010)
	nucleus	hepatocellular carcinoma cells	ND	ND	ND	ND, correlation with poor overall survival and large tumor size in hepatocellular carcinoma	(Ip *et al.*, 2007)
MMP-3	**cytosol**	dopaminergic neurons	ND	serine protease	ND	Induction of neuronal apoptosis upstream of caspase-3	(Choi *et al.*, 2008)
	cytoplasmic granules	dopaminergic neuroblastoma cells	ND	oxidative stress	α-syn	Increased fragment-induced aggregation and augmented toxicity of fragment-induced aggregates on cell viability	(Sung *et al.*, 2005)
	cytoplasm, nucleus	chondrosarcoma cells	CL- endocytosis, NLS, RAN-BP	ND	ND	Transcription of the *CTGF* gene, possibly affecting development, tissue remodeling and regeneration, arthritic and fibrotic diseases, and cancer progression	(Eguchi *et al.*, 2008)
	cytosol, nucleus	hepatocellular carcinoma cells, myofibroblasts, CHO cells	NLS	ND	ND	Induction of apoptosis	(Si-Tayeb *et al.*, 2006)

MMP-7	**cytoplasmic granules**	Paneth cells	ND	ND	pro-Crps, pro-CRSC	Antibacterial activity, contributing to clearance of intestinal infections	(Wilson *et al.*, 1999; Ayabe *et al.*, 2002; Shirafuji *et al.*, 2003; Shanahan *et al.*, 2010)
	Golgi apparatus	vaginal-cervical epithelial cells	secretion pathway	ND	occludin	Decrease of tight junction resistance	(Gorodeski, 2007)
	cytosol	cardiomyocytes	ND	ND	Cx43	Associated with adverse electrical remodeling and decreased survival after myocardial infarct	(Lindsey *et al.*, 2006)
	cytosol	neurons	CL-endocytosis	ND	SNAP-25	ND, possible perturbation of neurotransmitter exocytosis during brain inflammation	(Szklarczyk *et al.*, 2007b)
MMP-9	**secretory vesicles**	endothelial cells	secretion pathway	ND	ND	ND	(Nguyen *et al.*, 1998)
	secretory lysosomes, cytoskeleton, nucleus	astrocytes	endocytosis and ND	ND	ND	ND	(Sbai *et al.*, 2010)
	cytosol	lens fiber cells	ND	ND	βB1-, βB3- and γC-crys	Aggregation and precipitation leading to opacification of the eye lens and cataract	(Descamps *et al.*, 2005)
	sarcomere	cardiomyocytes	ND	oxidative stress	MHC	May contribute to the disorganization of the contractile apparatus in dilated cardiomyopathy	(Rouet-Benzineb *et al.*, 1999)
	mitochondria	cardiomyocytes	ND	ND	ND	May contribute to the exacerbation of myocyte mechanical dysfynction in hyperhomocysteinemia	(Moshal *et al.*, 2008)
	nucleus	neurons	ND	ND	PARP-1, XRCC1	Contribution to oxidative DNA damage in neurons after ischemic insult	(Yang *et al.*, 2010)
MMP-11	**TGN**	breast cancer cells	secretion pathway	furin	ND	ND	(Pei and Weiss, 1995; Santavicca *et al.*, 1996)
	ND		ND	alternative splicing	ND	ND	(Luo *et al.*, 2002)
MMP-12	**phagolysosome**	macrophages	ND	catalytic activity not required	/	Direct antimicrobial activity by disrupting bacterial cell membranes	(Houghton *et al.*, 2009)
MMP-13	**cytoplasm, nucleus**	neurons, astrocytes, oligodendrocytes	ND	ND	ND	ND, early consequence of cerebral ischemia	(Cuadrado *et al.*, 2009)
MMP-26	**cytoplasm**	breast and endometrial carcinoma cells	ND	autocatalysis	ERβ	May contribute to the disorganization of focal adhesions and actin filaments, resulting in changes in cell shape, adhesion and migration	(Savinov *et al.*, 2006)
	cytoplasm	prostate cancer cells	ND	autocatalysis	pro-MMP-9 pro-MMP-26	Putative intracellular activation, leading to enhanced invasiveness of prostate cancer cells	(Zhao *et al.*, 2003)
	cytoplasm, nucleus	cytotrophoblasts and choriocarcinoma cells	ND	autocatalysis	ND	ND, putative role in tissue-remodeling processes associated with placentation and tumor progression	(Zhang *et al.*, 2002)

MT1-MMP	**caveolae**	fibrosarcoma and endothelial cells	CAV-endocytosis	ND	ND	Mechanism of cell entry and/or relocalization to the leading edge of a migrating cell	(Puyraimond *et al.*, 2001; Remacle *et al.*, 2003; Galvez *et al.*, 2004)
	endosome, centrosome, pericentrosome	breast and colon carcinoma and glioma cells	endocytosis	furin, autocatalysis	PCNT	Induction of chromosome instability and aneuploidy promoting malignant transformation	(Golubkov *et al.*, 2006; Golubkov *et al.*, 2005b; Golubkov *et al.*, 2005a; Remacle *et al.*, 2005)
	cytosol, cytoskeleton	smooth muscle cells	ND	ND	FAK	May contribute to the disorganization of focal adhesions and actin filaments, resulting in changes in cell shape, adhesion and migration	(Shofuda *et al.*, 2004)
	nucleus	neurons	ND	furin	pro-MMP-2	Putative activation of MMP-2 wich contributes to oxidative DNA damage in neurons after ischemic insult	(Yang *et al.*, 2010)
	nucleus	hepatocellular carcinoma cells	CAV-endocytosis	ND	ND	ND, correlation with poor overall survival and large tumor size in hepatocellular carcinoma	(Ip *et al.*, 2007)
MT3-MMP	**cytosol, cytoskeleton**	smooth muscle cells	ND	ND	FAK	May contribute to the disorganization of focal adhesions and actin filaments, resulting in changes in cell shape, adhesion and migration	(Shofuda *et al.*, 2004)
	TGN	Madin-Darby canine kidney cells	secretion pathway	furin	ND	ND	(Kang *et al.*, 2002)
MT6-MMP	**PMN granules and secretory vesicles**	PMNs	secretion pathway	furin	ND	ND	(Kang *et al.*, 2001)
TIMP-1	**cytoplasm**	fibroblasts	ND	/	/	ND, continous accumulation during cell cycle	(Zhao *et al.*, 1998)
	nucleus	fibroblasts	ND	/	/	ND, cell cycle-dependent accumulation reaching a maximum in the S phase	(Zhao *et al.*, 1998)
	nucleus	breast carcinoma cells	ND	/	/	ND	(Ritter *et al.*, 1999)
	nucleus	chondrosarcoma cells	ND	/	/	ND, putative control of nuclear MMP-3 proteolytic activity to enable proper transcription factor activity	(Si-Tayeb *et al.*, 2006)
TIMP-2	**cytoplasm**	fibroblasts	ND	/	/	ND	(Zhao *et al.*, 1998)
TIMP-3	**cytoplasm**	fibroblasts	ND	/	/	ND	(Zhao *et al.*, 1998)
TIMP-4	**sarcomere**	cardiomyocytes	ND	/	/	ND, putative regulatory role in cardiomyocyte homeostasis	(Schulze *et al.*, 2003)
	nucleus	cytotrophoblasts and choriocarcinoma cells	ND	/	/	ND, putative regulation of MMP-26 in tissue-remodeling processes associated with placentation and tumor progression	(Zhang *et al.*, 2002)

CAV-endocytosis, caveolae-dependent endocytosis; **CHO**, Chinese hamster ovary; **CL-endocytosis**, clathrin-dependent endocytosis; **ND**, not defined; **NLS**, nuclear localization signal; **PMN**, polymorphonuclear leukocytes; **RAN-BP**, RAN-binding protein; **RPE,** retinal pigment epithelial; **TGN**, *trans*-Golgi network.

*The meaning of the acronyms can be found in Table 5.

Of interest, Si-Tayeb and coworkers found two activated forms of MMP-3 in the nucleus of hepatocellular carcinoma cells and myofibroblasts, whereas pro-MMP-3 was not found in the nucleus (Si-Tayeb *et al.*, 2006). Using the bioinformatics software PSORT (Nakai and Horton, 1999), they detected a putative NLS (PKWRKTH) at position 107 to 113 in the catalytical domain, which was well conserved between the human, mouse and rat sequences of MMP-3. Nuclear entry was shown to be dependent on this NLS, as deletion of 2 amino acids led to a large decrease in nuclear localization. Since pro-MMP-3 was not found in the nucleus, the NLS may be shielded by the propeptide, much like the catalytical cleft is protected in the latent pro-MMPs. In contrast, pro-MMP-2 carries a putative NLS on its COOH-terminus that should be accessible without pro-peptide removal, thus allowing transfer of the full-length protein to the nucleus. Nuclear translocation of MMP-3 was confirmed by Eguchi and colleagues, who showed that extracellular MMP-3 is taken up into chondrosarcoma cells and subsequently translocates to the nucleus where it induces transcription of the *connective tissue growth factor (CTGF)* gene (*vide infra*) (Eguchi *et al.*, 2008). Five additional NLSs were found, three in the pro-domain of MMP-3 and two in the hemopexin domain, that were all driving an enhanced green fluorescent protein (EGFP)-NLS construct to the nucleus. In addition, MMP-3 was associated with a RAN-binding protein, which was involved in the nuclear import. Hence, nuclear entry of MMP-3 is regulated by two independent pathways. Loss of nuclear MMP-3 was detected 30 min after nuclear entry, suggesting degradation or nuclear export to the cytoplasm by a Leu-rich NES sequence in the hemopexin domain.

Besides MMP-2 and MMP-3, Si-Tayeb and colleagues detected putative NLSs in the sequences of MMP-1,-8,-10,-13,-19,-20,-23 and MT1-, MT3-, MT4- and MT5-MMP, which suggests that nuclear entry may be a feature of many MMPs. MMPs that do not contain an NLS may enter the nucleus by binding to cargoes, such as RAN-binding proteins, other proteins with an NLS, various types of RNA and complexes of RNA plus proteins (ribonucleoproteins or RNPs) (Quimby and Corbett, 2001). For example, MMP-9 may enter the nucleus bound to the nuclear protein Ku, an interaction that was detected at the cell surface (Monferran *et al.*, 2004). In addition, some proteins without NLS but associated with clathrin-coated pits or caveolae are also able to shuttle in and out of the nucleus, albeit the precise mechanisms involved in these processes are poorly understood (Benmerah *et al.*, 2003; Lee *et al.*, 2004a). Such nuclear translocation *via* caveolae-mediated endocytosis was proposed for MT1-MMP in aggressive hepatocellular carcinoma cells (Ip *et al.*, 2007).

To avoid excessive proteolysis of nuclear proteins during cellular homeostasis, these nuclear MMPs may be under inhibition by TIMP-1 and TIMP-4, which are also present in the nucleus (*cf.* Table 4) (Zhao *et al.*, 1998; Ritter *et al.*, 1999; Zhang *et al.*, 2002; Si-Tayeb *et al.*, 2006)

In conclusion, a systematic investigation of the localization mechanisms and the presence of MMPs in the various subcellular compartments - under conditions of cellular stress and homeostasis - may provide more insight into the present fragmented image. In addition, this may contribute to the elucidation of intracellular functions of MMPs, which may be of great importance when considering MMP inhibition therapy.

3.3 Intracellular proteolysis by MMPs in physiology and pathology

It has been clearly demonstrated in the previous chapters that MMPs may be activated intracellularly or enter cells and various cellular compartments after extracellular activation. This leads to questions about the physiological and pathological implications of intracellular MMP activity. Intracellular proteins, which are the subject of intracellular MMP proteolysis, will be discussed in the context of the physiological or pathological pathways affected by their cleavage. We will review well-established, confirmed intracellular substrates, which are summarized in Table 5 with the (putative) location of their cleavages. This table also includes the biological roles of the substrates, the studied MMPs, the identified cleavage sites, the context of the cleavage (*in vitro*, *ex vivo* or *in vivo*) and the (possible) effects of the cleavages on physiopathology. Cleavage of substrates identified by degradomics (*cf.* Table 3) will be mentioned in the relevant sections.

3.3.1 Intracellular proteolysis in innate immune defense

The efficacy of innate host defense can be attributed to the ability of the immune system to recognize and neutralize microbial invaders quickly and efficiently. In mammals, inflammatory leukocytes and affected epithelia synthesize and/or mobilize anti-microbial peptides that are capable of directly killing a variety of pathogens, i.e. cathelicidins and defensins. The α- and β-defensins comprise a family of cationic trisulfide peptides that kill bacteria by membrane disruption. α-defensins were identified as antimicrobial proteins purified from extracts of cytoplasmic granules of polymorphonuclear (PMN) leukocytes. Mice, however, lack leukocytic defensins. Nevertheless, Paneth cells that populate the crypts of Lieberkühn throughout the mouse small intestine express many antimicrobial α-defensins called cryptdins (crypt defensins). Cryptdin peptides, packaged in apically oriented granules of Paneth cells, are secreted into the crypt by degranulation that is both constitutive at a base-line level and inducible by infection. Biosynthesis of prepro-α-defensins (~10 kDa) involves the rapid cleavage of the signal peptide producing pro-α-defensins (~8.5 kDa) that have little or no microbicidal acitivity *in vitro*. Activation of defensin peptides requires

proteolytic removal of the NH_2-terminal acidic pro-region, yielding a mature peptide of ~3.5 kDa (Selsted and Ouellette, 2005).

MMP-7 colocalizes with cryptdins (Crps) in mouse Paneth cells and mediates the processing and activation of various Crps *in vitro* (Wilson *et al.*, 1999). MMP-7 cleaves pro-Crp-1, -6 and -15 at Ser43-Val44; Ser53-Leu54 and Ser58-Leu59. The latter cleavage generates the NH_2-terminal consensus sequence in all related pro-Crps (pro-Crp-1 to -16) except for pro-Crp-4 and -5, which do not show sequence identity for the above mentioned cleavage sites (Putsep *et al.*, 2000). However, they are cleaved at the same positions, i.e. Ser43-Ile44, Ala53-Leu54 and Ser58-Leu59 (Ayabe *et al.*, 2002; Shirafuji *et al.*, 2003). When analyzing the processing intermediates of Crp4 ($Crp4^{44-92}$, $Crp4^{54-92}$ and $Crp4^{59-92}$), the most bactericidal of the known mouse α-defensins, Weeks *et al.* found that their *in vitro* antibacterial effects are very similar, contrasting with the lack of bactericidal and membrane-disruptive capacity of pro-$Crp-4^{20-92}$. Hence, cleavage by MMP-7 at Ser43-Ile44 is sufficient to activate bactericidal activity, and the amino acids between 20 and 43 of the proregion maintain the precursor in an inactive state, which may be important to prevent deleterious effects to the host cell during pro-α-defensin synthesis, folding and packaging into granules (Weeks *et al.*, 2006). Why this processing occurs intracellularly is an enigma, but intragranular colocalization of bactericidal peptides and MMP-7 might enhance their cleavage, when compared to cleavage in the diluted extracellular milieu. In addition, these and other observations (*vide infra*) do not exclude the possibility of additional MMP-7-mediated extracellular Crp activation.

MMP-7 gene deletion ablates pro-Crp processing, resulting in accumulation of cryptdin precursors in Paneth cell granules and the absence of activated mature Crp peptides in the small intestine. Since Crps constitute about 70% of the bactericidal activity released by Paneth cells (Ayabe *et al.*, 2000), MMP-7 knockout mice without functional Crp peptides, have a functional defect in clearance of intestinal infections, and they succumb more rapidly and to lower doses of virulent *Salmonella typhimurium* compared with control mice (Wilson *et al.*, 1999). Thus, defective pro-Crp activation is associated with a deficit in mucosal immunity and increased risk of systemic disease.

Whereas murine leukocytes do not contain defensin peptides, in humans, α-defensins are primarily found in neutrophils (human neutrophil peptides 1-4 or HNP-1 to -4) and small intestinal Paneth cells (human defensins 5 and 6 or HD-5 and -6), the latter being activated by trypsin (Ghosh *et al.*, 2002). MMP-7 cleaves within the prodomain of pro-HNP-1, which does not produce the sequence of the mature peptide, but of a processing intermediate with antimicrobial activity (Wilson *et al.*, 2009). However, since MMP-7 is not present in neutrophils, and the neutrophilic MMPs (MMP-8, MMP-9 and MT6-MMP) are compartmentalized separately from α-defensins, proteolytic activation of pro-HNPs within maturing human neutrophil granules does not seem to be an MMP-dependent process.

In the mouse, α-defensin genes duplicated and diversified further to give rise to the *Defcr-rs* gene subfamily that codes for numerous cysteine-rich sequence 4C (CRS4C) peptides that are unique to mice. Remarkably, preproregions of CRSC4 and Crp peptides are nearly identical but the mature CRSC4 peptides are cysteine-rich peptides very different from Crps (Ouellette and Lualdi, 1990). Native CRSC4 peptides purified from mouse small intestine exist as disulfide-stabilized homodimers and heterodimers with antibacterial activity (Huttner and Ouellette, 1994). As expected from the high sequence identity of the proregions, inactive pro-CRSC4-1 (cryptdin-related protein 4C-1, defensin-related cryptdin-related sequence 2) was found to be converted to mature bactericidal CRSC4-1 by MMP-7-mediated *in vitro* proteolysis of the precursor proregion and at the same residue positions as determined for Crp activation, i.e. Ser43-Val44, Ala53-Leu54 and Ala58-Leu59. The absence of processed CRSC4 peptides in MMP-7 knockout mice demonstrates the *in vivo* activation of CRS4C by MMP-7 (Shanahan *et al.*, 2010).

Besides activation of antimicrobial peptides, it was recently discovered that MMPs may function as antimicrobial agents themselves. A recent study showed that MMP-12 possesses antimicrobial activities that do not require the catalytical domain, but reside in the COOH-terminal hemopexin domain. The hemopexin domain of MMP-12 inhibited the growth of various bacterial strains *in vitro*. The origin of bactericidal activity was narrowed down to a unique four amino acid sequence of acidic amino acids flanked by basic residues (e.g. Lys348-Asp-Asp-Lys in human MMP-12) present on an exposed β loop of the protein. This sequence is homologous in rabbit, rat, mouse and human MMP-12, but is not present in any other MMP. MMP-12-deficient mice exhibited impaired bacterial clearance and increased mortality when challenged with both Gram-negative and Gram-positive bacteria at macrophage-rich portals of entry, such as the peritoneum and the lung. After engulfment of bacteria by macrophages, intracellular stores of MMP-12 were mobilized to macrophage phagolysosomes, as determined by immunogold electron microscopy. Once inside phagolysosomes, MMP-12 adhered to bacterial cell walls and used its antimicrobial peptide to kill the bacteria by disruption of the cell membranes (Houghton *et al.*, 2009). *Strictu sensu*, in this case no evidence is provided for a host cell substrate conversion, but instead MMP-12 hemopexin domain binds to and kills micro-organisms by a non-catalytical function.

In conclusion, both catalytical and non-catalytical actions of MMPs inside cells may contribute to the host's innate immune defense against microorganisms.

Table 5. Confirmed intracellular MMP substrates and the (putative) physiopathological effects of their cleavage

Substrate symbol	Substrate name	Subcellular localization	Biological role substrate	MMP	Cleavage sites*	Physiological/pathological effect of the cleavage	References
CYTOSOL							
βB1-crys	βB1-crystallin	cytosol	Prevention of protein unfolding or aggregation, structural component of the eye lens	MMP-9	Ala47-Lys48^{m} *In vitro + ex vivo + in vivo*	Aggregation and precipitation leading to opacification of the eye lens and cataract	(Descamps *et al.*, 2005)
βB3-crys	βB3-crystallin	cytosol	Prevention of protein unfolding or aggregation, structural component of the eye lens	MMP-9	*Ex vivo*	Aggregation and precipitation leading to opacification of the eye lens and cataract	(Descamps *et al.*, 2005)
γC-crys	γC-crystallin	cytosol	Prevention of protein unfolding or aggregation, structural component of the eye lens	MMP-9	*In vitro*	ND, putative contribution to opacification of the eye lens and cataract	(Descamps *et al.*, 2005)
Cx43	connexin-43	cytosol, mitochondria	Gap junction protein	MMP-7	*In vitro + in vivo*	Associated with adverse electrical remodeling and decreased survival after myocardial infarction	(Lindsey *et al.*, 2006)
ERβ	estrogen receptor-β	cytosol, nucleus	Transmission of 17β-estradiol signaling	MMP-26	*In vitro + in vivo*	ND, associated with a favorable prognosis and longer overall survival of breast cancer patients	(Savinov *et al.*, 2006)
GSK-3β	glycogen synthase kinase-3β	cytosol, mitochondria, nucleus	Multifunctional Ser/Thr kinase regulating cellular functions such as apoptosis, cell cycle, cell migration and gene expression	MMP-2	*In vitro*	Augmentation of GSK-3β kinase activity, which may contribute to apoptosis and cardiac injury resulting from enhanced oxidative stress	(Kandasamy and Schulz, 2009)
SNAP-25	synaptosomal-associated protein of 25 kDa	cytosol	Neurovesicular fusion and neurotransmitter release	MMP-7	Ala128-Ile129 *In vitro*	ND, possible perturbation of neurotransmitter exocytosis during brain inflammation	(Szklarczyk *et al.*, 2007b)
ZO-1	zona occludens-1	cytosol, nucleus	Tight junction scaffolding protein mediating cytoskeletal anchorage of the tight junction	MMP-9 MMP-13	*In vitro + in vivo*	BBB disruption in cerebral ischemia	(Harkness *et al.*, 2000; Asahi *et al.*, 2001; Lu *et al.*, 2009)
CYTOPLASMIC GRANULES							
pro-Crp-1-3	pro-Cryptdin-1 to -3	cytoplasmic Paneth granules	Bactericidal action by bacterial membrane disruption	MMP-7	Ser43-Val44^{m} Ser53-Leu54^{m} Ser58-Leu59^{m} *In vitro + in vivo*	Antibacterial activity, contributing to clearance of intestinal infections	(Ayabe *et al.*, 2002; Shirafuji *et al.*, 2003; Wilson *et al.*, 1999)
pro-Crp-4-5	pro-Cryptdin-4,-5	cytoplasmic Paneth granules	Bactericidal action by bacterial membrane disruption	MMP-7	Ser43-Ile44^{m} Ala53-Leu54^{m} Ser58-Leu59^{m} *In vitro + in vivo*	Antibacterial activity, contributing to clearance of intestinal infections	(Ayabe *et al.*, 2002; Shirafuji *et al.*, 2003; Wilson *et al.*, 1999)
pro-Crp-6-16	pro-Cryptdin-6 to -16	cytoplasmic Paneth granules	Bactericidal action by bacterial membrane disruption	MMP-7	Ser43-Val44^{m} Ser53-Leu54^{m} Ser58-Leu59^{m} *In vitro + in vivo*	Antibacterial activity, contributing to clearance of intestinal infections	(Ayabe *et al.*, 2002; Shirafuji *et al.*, 2003; Wilson *et al.*, 1999)

pro-CRS4C	cysteine-rich sequence 4C	cytoplasmic Paneth granules	Bactericidal action by bacterial membrane disruption	MMP-7	Ser43-Val44[m] Ala53-Leu54[m] Ala58-Leu59[m] *In vitro + in vivo*	Antibacterial activity, contributing to clearance of intestinal infections	(Shanahan *et al.*, 2010)
α-syn	α-synuclein	cytoplasm, mitochondria, nucleus	Regulation of neurotransmitter release, pathogenic accumulation and aggregation in Lewy bodies in Parkinson's disease	MMP-3	Thr54-Val55 Ala90-Ala91 Glu57-Lys58 Ala91-Thr92 Ala78-Gln79 Thr92-Gly93 Gln79-Lys80 Gly93-Phe94 Lys80-Thr81 Phe94-Val95 Ser87-Ile88 Lys102-Asn103 *In vitro*	Increased fragment-induced aggregation and augmented toxicity of fragment-induced aggregates on cell viability; inhibition of aggregation with high MMP concentrations	(Sung *et al.*, 2005; Levin *et al.*, 2009)
				MMP-1	Gly7-Leu8 Val71-Thr72 Ala18-Ala19 Ala78-Gln79 Lys21-Thr22 Ala90-Ala91 Gly41-Ser42 Lys97-Asp98 Gly47-Val48 Tyr33-Gln134 Val70-Val71 Gln134-Asp135 *In vitro*	Increased aggregation after limited proteolytis, inhibition of aggregation with high MMP concentrations	(Sung *et al.*, 2005; Levin *et al.*, 2009)
				MMP-9	Phe4-Met5 Val71-Thr72 Gly7-Leu8 Gly73-Val74 Ala18-Ala19 Val74-Thr75 Val66-Gly67 Ala78-Gln79 Val70-Val71 Asp98-Gln99 *In vitro*	No significant increase in aggregation after limited proteolytis, inhibition of aggregation with high MMP concentrations	(Sung *et al.*, 2005; Levin *et al.*, 2009)
				MT1-MMP MMP-2	*In vitro*	ND	(Sung *et al.*, 2005)
CYTOSKELETON							
α-actinin	α-actinin	cytoskeleton, myofibril, sarcomere, Z-disc, nucleolus, extracellular	Connection of actin filaments of adjacent sarcomeres and transmission of the force generated by the actin-myosin complex	MMP-2	*In vitro + ex vivo*	ND, may contribute to cardiac dysfunction induced by peroxynitrite	(Sung *et al.*, 2007)
Desmin	desmin	cytoplasm, cytoskeleton, Z-disk	Intermediate filament protein involved in cellular resistance to external stress	MMP-2	*In vitro*	ND	(Sung *et al.*, 2007)
FAK	125 kDa focal adhesion kinase	cytoplasm, cytoskeleton	Tyrosine kinase involved in cytoskeleton remodeling, formation and disassembly of cell adhesion structures and regulation of Rho-family GTPases	MT1-MMP MT3-MMP	*In vitro*	May contribute to the disorganization of focal adhesions and actin filaments, resulting in changes in cell shape, adhesion and migration	(Shofuda *et al.*, 2004)
Pericentrin	pericentrin	centrosome	Integral centrosomal protein required for normal centrosome functioning and mitotic spindle formation	MT1-MMP	Gly1156-Leu1157 Gly672-Leu673 Ser2068-Leu2069[c] *In vitro*	Induction of chromosome instability and aneuploidy promoting malignant transformation	(Golubkov *et al.*, 2006; Golubkov *et al.*, 2005b)

SARCOMERE							
MHC	myosin heavy chain	sarcomere	Mechanical protein that generates force during muscle contraction	MMP-9 MMP-2	*In vitro*	May contribute to the disorganization of the contractile apparatus in dilated cardiomyopathy	(Rouet-Benzineb *et al.*, 1999)
MLC-1	myosin light chain-1	cytoplasm, cytoskeleton, sarcomere	Mechanical protein that generates force during muscle contraction	MMP-2	*In vitro* + *ex vivo*	Contributes to mechanical dysfunction of the heart after ischemia-reperfusion injury	(Sawicki *et al.*, 2005)
TnI	troponin I	sarcomere	Binds to actin in thin filaments to hold the troponin-tropomyosin complex in place and inhibits myocardial force generation in the resting phase	MMP-2	*In vitro* + *ex vivo*	Contributes to mechanical dysfunction of the heart after ischemia-reperfusion injury and cytokine-induced myocardial dysfunction	(Wang *et al.*, 2002a; Gao *et al.*, 2003)
NUCLEUS							
PARP-1	poly (ADP-ribose) polymerase-1	nucleus	ATP-dependent DNA repair enzyme	MMP-2	*In vitro*	Contribution to oxidative DNA damage in neurons early after ischemia, putative removal of excess PARP-1 during later stages of cardiac and cerebral ischemia, sparing the cell from ATP depletion	(Kwan *et al.*, 2004)
XRCC1	X-ray cross-complementary factor 1	nucleus	Central role in the DNA base excision repair pathway by interaction with the DNA repair enzymes	MMP-2 MMP-9	*In vitro* + *in vivo*	Contribution to oxidative DNA damage in neurons after ischemic insult	(Yang *et al.*, 2010)
EXTRACELLULAR MILIEU							
αB-crys	αB-crystallin	cytosol, cytoskeleton, nucleus	Prevention of protein unfolding or aggregation, structural component of the eye lens	MMP-9	*Cf.* Table 8	Generation of immunodominant T cell epitopes and removal of the neuroprotective effects of αB-crys in multiple sclerosis	(Starckx *et al.*, 2003)
CAP1	adenylyl cyclase-associated protein 1	cytoskeleton	Enhancement of actin filament turnover; roles in cell morphology, migration and endocytosis; promotion of apoptotis; autoantigen in SLE and RA	MMP-9 MMP-2,-8, -13	*In vitro* + *in vivo*	ND, putative anti-apoptotic effect	(Cauwe *et al.*, 2008)
Gelsolin	gelsolin	cytosol, cytoskeleton, secreted	F-actin capping and severing; nucleation of F-actin assembly; scavenging of actin and pro-inflammatory components in the plasma; defects are the cause of amyloidosis type 5 or familial amyloidosis Finnish type	MMP-3	Asn416-Val417 Ser51-Met52 Ala435-Gln436 *In vitro*	May cause actin toxicity by weakening the actin scavenger system; generation of amyloidogenic peptides that cause familial amyloidosis Finnish type; may abolish the anti-apoptotic and Aβ-reducing function of gelsolin in Alzheimer's disease	(Hwang *et al.*, 2004; Park *et al.*, 2006; Page *et al.*, 2005; Antequera *et al.*, 2009; Ray *et al.*, 2000)
				MT1-MMP	Ala242-Met243 *In vitro*		
				MMP-1,-2, -7,-9	*In vitro*		

*Cleavage sites are indicated in the human sequence. The suffix in superscript indicates an alternative organism: m, murine; c, canine.

NB, MMPs that were described to be activated intracellularly by other MMPs are considered as intracellular substrates but are not included in this table.

3.3.2 Intracellular proteolysis in cancer

Extracellular proteolysis by MMPs has conflicting effects on cancer cell differentiation, proliferation, escape from apoptosis and immune surveillance, migration and invasion, and tumor angiogenesis. Depending on the substrates, as well as on the specific stage of cancer cell progression, the same MMP may exert a cancer-promoting or -limiting function (Egeblad and Werb, 2002; Cauwe *et al.*, 2007). The stepwise progression from an early dysplastic lesion to full-blown metastatic malignancy is associated with increases in genomic instability (Vogelstein and Kinzler, 2004). Chromosome instability, the inability to correctly segregate sister chromatids during mitosis (leading to an abnormal chromosome number termed aneuploidy), is thought to initiate and propagate malignant transformation (Schvartzman *et al.*, 2010). Dysruption of centrosome structure is an integral aspect of the origin of chromosomal instability in many cancers. Centrosomes nucleate and organize microtubules and form spindle poles during mitosis. In normal cells, the centrosome is usually composed of a pair of barrel-shaped structures, the centrioles, which are embedded in a lattice-like pericentriolar material (Zyss and Gergely, 2009). As discussed before, cell surface-associated MT1-MMP is internalized by endocytosis, and microtubular trafficking causes the protease to accumulate in the centrosomal and pericentrosomal compartment (Remacle *et al.*, 2003; Remacle *et al.*, 2005). MT1-MMP expression is associated with tumor progression and metastasis and is implicated both in the breaching of basement membranes by tumor cells and invasion of the ECM. In addition, MT1-MMP activates soluble MMPs, cleaves adhesion and signaling receptors and acts as a tumor growth factor (Poincloux *et al.*, 2009). Besides these extracellular invasion-promoting functions, MT1-MMP was shown to have an intracellular oncogenic function by cleaving the integral centrosomal protein, pericentrin, at Gly672-Leu673 and Gly1156-Leu1157 (Golubkov *et al.*, 2005a; Golubkov *et al.*, 2005b). Pericentrin and pericentrin-2 (pericentrin-B or kendrin) are derived from splice variants of the same gene and are known to be essential for normal centrosome function by the anchorage of the γ-tubulin ring complex - that initiates microtubule nucleation - to the centrosome (Takahashi *et al.*, 2002). Normal epithelial cells transfected with MT1-MMP acquired the ability to activate MMP-2, to cleave pericentrin and to invade a Matrigel matrix. These events were associated with aberrations in chromosome segregation, up-regulation of gene expression of multiple oncogenic genes, aneuploidy and transformation of non-malignant human mammary epithelial cells. Indeed, MT1-MMP-expressing human epithelial cells were efficient at generating tumors in the orthotopic xenograft model in immunodeficient mice. However, the tumors regressed because of insufficient host angiogenic response and inadequate neovascularization, also in line with the lack of upregulation of pro-angiogenic gene expression. Both human and canine pericentrin peptides are cleaved by MT1-MMP *in vitro*, the latter at the Ser2068-Leu2069 peptide bond. However, murine pericentrin is resistant to cleavage because of an Asp residue at the P1 position (*cf.* Figure 4) instead of Ser or Gly. In addition, tumor biopsies showed proteolytic pericentrin fragments in association with high levels of activated MT1-MMP, whereas normal tissues contained intact pericentrin. Hence, the intracellular proteolysis of pericentrin by MT1-MMP may have an important role in the generation of chromosome instability and malignant transformation (Golubkov *et al.*, 2006; Golubkov *et al.*, 2005b; Golubkov *et al.*, 2005a).

Once tumor cells have acquired invasive capacities, polarized motility is governed by the organization of a leading edge in the direction of cell movement. Cell motility requires molecular processes at the cell surface in which contacts between the invading tumor cell and the surrounding cells and stroma are repeatedly broken (anti-adhesion) and new contacts are established as the tumor cell moves forward (adhesion). Hence, the leading edge is stabilized by the formation of new focal adhesions or cell-ECM contact sites. Besides its pericellular proteolytic functions, MT1-MMP also seems to affect tumor cell migration by perturbation of focal adhesions. Indeed, in vascular smooth muscle cells, overexpression of MT1-MMP (and MT3-MMP) results in cell rounding, decreased adherence and increased migration (Shofuda *et al.*, 2001). These events were associated with a decreased number of focal contacts with integrin-mediated adhesion, whereas the cell surface expression of integrin subunits remained unchanged, excluding their cleavage by MT1-MMP. However, MT1-MMP overexpression resulted in the cleavage of the 125 kDa focal adhesion kinase (FAK, pp125FAK, protein-tyrosine kinase 2) into a 90 kDa NH_2-terminal fragment (Shofuda *et al.*, 2004). FAK is a cytoplasmic protein-tyrosine kinase recruited to and activated at focal adhesion sites and is especially important for the connection of ECM-integrin complexes with downstream signaling molecules and actin stress fibers (Tomar and Schlaepfer, 2009). FAK cleavage was associated with partial dissociation of paxillin from the integrin-FAK complex and both events were inhibited by the metalloproteinase inhibitor BB94. Paxillin is another important cytoskeletal and scaffolding protein recruited early to focal adhesions (Tomar and Schlaepfer, 2009). Loss of the FAK/paxillin interaction may be a major contributing factor in the reduced organization of focal adhesions and actin filaments, resulting in decreased integrin-mediated cell adhesion observed in cells overexpressing MT1-MMP. Cleavage of FAK may be induced by MT1-MMP through an indirect mechanism, since calpains, caspases and granzyme B were also shown to proteolyse FAK (Carragher *et al.*, 1999; Wen *et al.*, 1997; Gervais *et al.*, 1998). However, as MT1-MMP was already mentioned to have an intracellular cleavage function in tumor cells, and both MT1-MMP and MT3-

MMP were found in the cytoskeletal fraction of vascular smooth muscle cells (Shofuda *et al.*, 2004), it is very likely that FAK is a direct intracellular target of MT1-MMP. Interestingly, viral Src kinase-transformed cells activate a FAK-dependent mechanism that attenuates endocytosis of MT1-MMP (*cf.* 3.2.1). This in turn increases cell-surface expression of MT1-MMP and pericellular degradation of the ECM (Wu *et al.*, 2005). Hence, intracellular cleavage of FAK by MT1-MMP may be a way to loosen focal adhesions, permitting the cell to detach for further migration, and at the same time it may enhance endocytosis of MT1-MMP at the rear end for recycling and relocalization at the leading edge. In this context, a membrane flip-flop of MT1-MMP, as suggested in section 3.2.1, would allow MT1-MMP to cleave FAK and other cytoskeletal substrates at the focal adhesion (*cf.* Tables 3 and 5).

Altogether, this may suggest a bimodal role for MT1-MMP in cancer progression. The early function of MT1-MMP takes place inside cells and promotes malignant transformation. When the cells have acquired invasive capacity, MT1-MMP is delivered to the surface of invadipodia where it assists in the proteolysis of a path for the migrating cell. After migration the intracellular cleavage of FAK may loosen focal adhesions at the rear end and recycle MT1-MMP to the novel invadipodia. Hence, MT1-MMP is not just a protease that supports tumor growth and metastasis, but it also functions as an oncogene that promotes chromosome instability and malignant transformation of normal cells at the early stages of the transition to malignancy (Golubkov and Strongin, 2007). Clinical trials with MMP inhibitors for the treatment of metastatic cancer have generally failed (Coussens *et al.*, 2002). Indeed, the beneficial effects of MT1-MMP inhibition in animal models of cancer may be explained in part by the absence of these oncogenic functions, which will not be the case for MMP inhibition in late-stage cancer trials.

Besides its actions in the centrosomal compartment and at focal adhesions, activated MT1-MMP is also detected in the nuclei of hepatocellular carcinoma cells (*cf.* 3.2.6). Of interest, cancer patients with nuclear MT1-MMP (and co-localized MMP-2) have a poor overall survival and large tumor size, whereas MT1-MMP is not found in nuclei of the normal paralleled liver tissues and normal control livers (Ip *et al.*, 2007). Since the protein composition of the nuclear matrix is altered in cancer cells (Zink *et al.*, 2004), it is tempting to speculate that MT1-MMP, MMP-2 and other nuclear MMPs (*cf.* 3.2.6) may contribute to the degradation of nuclear matrix proteins. Firm proof-of-principle is not available but specific proteins in the nuclear matrix, e.g. fibronectin, are known extracellular substrates of MMPs.

Another MMP that may affect cancer progression by its nuclear function is MMP-3. As mentioned in section 3.2.6 and Table 4, activated MMP-3 was found in the nuclei of cultured chondrocytes, in normal and osteoarthritic chondrocytes (Eguchi *et al.*, 2008), in the nuclei of dopaminergic neurons (Choi *et al.*, 2008) and hepatocytes (Si-Tayeb *et al.*, 2006). In the latter two studies, nuclear MMP-3 was found to promote apoptosis, which will be discussed in 1.4.5. In chondrocytes, nuclear MMP-3 interacts with the transcription enhancer dominant in chondrocytes (TRENDIC) in the *connective tissue growth factor* (*CTGF*) gene promoter and activates *CTGF* gene transcription. Of interest, both the catalytical domain and the hemopexin domain can activate the *CTGF* promoter independently (Eguchi *et al.*, 2008). CTGF (hypertrophic chondrocyte-specific protein 24, insulin-like growth factor-binding protein 8, CCN family member 2) is a member of the CCN family of secreted, matrix-associated proteins encoded by immediate early genes that play various roles in angiogenesis and tumor growth (Chu *et al.*, 2008). CCN stands for **c**ysteine-rich 61, **c**onnective tissue growth factor and **n**ephroblastoma overexpressed. CTGF is a multifunctional signaling modulator involved in a wide variety of biological and pathological processes such as angiogenesis, osteogenesis, renal disease, skin disorders and tumor development. Hence, MMP-3-induced transcription of *CTGF* in chondrocytes may be involved in matrix diseases, e.g. osteoarthritis and rheumatoid arthritis, and in fibrotic diseases, such as systemic sclerosis and atherosclerosis (Eguchi *et al.*, 2008). CTGF regulates cancer cell migration, invasion, angiogenesis and anoikis. Although CTGF expression is mostly associated with progression of many kinds of cancers, its role may vary considerably, depending on the tissue involved (Chu *et al.*, 2008). CTGF in turn induces increased transcription of the *MMP-1,-2,-3,-7,-9* and *MT1-MMP* genes, but reduces transcription of *TIMP-1* and *TIMP-2* (Kondo *et al.*, 2002; Chen *et al.*, 2001a), which may then result in a positive feedback loop in angiogenesis and tumor progression. In addition, CTGF is cleaved by MMP-1,-2,-3,-7,-13 and MT1-MMP (Hashimoto *et al.*, 2002; Butler *et al.*, 2008; Dean *et al.*, 2007; Dean and Overall, 2007), which may terminate the amplification loop.

Angiogenesis is an important mechanism in tumor biology. MMPs, and MMP-9 in particular, are involved in various aspects of the angiogenic switch (Hanahan and Weinberg, 2000) and in the angiogenic process (Ardi *et al.*, 2007; Deryugina and Quigley, 2010). So far, most validated substrates, e.g. CTGF, are extracellular proteins. A considerable amount of putative intracellular substrates of MMPs are linked to endothelial cell function (*cf.* Tables 3 and 5). However, the biological relevance of the cleavage of these intracellular proteins in the process of angiogenesis needs further exploration.

In contrast with these tumor-promoting functions of intracellular MMPs, MMP-26 may have antitumor properties inside breast cancer cells. MMP-26 is characterized by an unorthodox, autolytic activation mechanism (*cf.* 1.1) and accumulates primarily inside cells, as only a small fraction of the enzyme is secreted into the extracellular milieu (Strongin, 2006). In contrast to other MMPs, the promoter of the *MMP-26* gene includes the estrogen-response element (ERE). The estrogen-estrogen receptor (ER) complex stimulates the transcriptional activity of the *MMP-26* gene promoter in hormone-regulated carcinomas *via* binding of the ERE motif (Li *et al.*, 2004). Interestingly, estrogen receptor β (ERβ, nuclear receptor subfamily 3 group A member 2) is susceptible to proteolysis by MMP-26, whereas ERα is resistant. MMP-26 targets the NH_2-terminal region of ERβ coding for the divergent NH_2-terminal A/B domain that is responsible for the ligand-independent transactivation function *in vitro*. In breast carcinoma specimens, the ERα-dependent expression of MMP-26 correlated inversely with the residual levels of intact ERβ in the cytoplasm, as determined by immunohistochemical analysis. The levels of MMP-26 are low in the normal mammary epithelium but are strongly upregulated in ductal carcinoma *in situ* (DCIS). However, during further disease progression the expression of MMP-26 decreases again. In contrast with many tumor-promoting MMPs, the expression of MMP-26 in DCIS is correlated with a longer overall patient survival (Savinov *et al.*, 2006).

On the contrary, activation of pro-MMP-9 by MMP-26 promotes invasion of human prostate cancer cells (Zhao *et al.*, 2003). Activated forms of MMP-9 were shown to accumulate in the cytosol of basal and phorbol PMA-stimulated human endothelial cells. Whereas MMP-9 was found complexed to TIMP-1 in the conditioned medium, it existed as a free enzyme in membrane-bound vesicles that were especially prominent at the invadipodia (as determined with immunogold electron microscopy). In contrast, TIMP-1 was spread more diffusely throughout the cytoplasm, occasionally present in small vesicles but never in the MMP-9-containing vesicles (Nguyen *et al.*, 1998). Both MMP-3, which was also detected in the cytoplasm, and self-activated MMP-26 may act as activators for the intracellular pro-MMP-9 (*cf.* 1.1). Storage of intracellularly activated MMP-9, ready for rapid release, may facilitate invasion and migration of cancer cells.

Finally, MMP-1 was found to be strongly associated with mitochondrial membranes (*cf.* 3.2.5*)* and nuclei and accumulated within cells during the mitotic phase of the cell cycle. The intracellular association of MMP-1 to mitochondria and nuclei conferred resistance to apoptosis (*vide infra*), which may be a mechanism for tumor cells to escape from apoptosis (Limb *et al.*, 2005).

3.3.3 Intracellular proteolysis in cardiac disease

Heart failure is a common cause of morbidity and mortality with a high incidence in developed countries. Approximately every 25 seconds, an American will have a cardiac event, and approximately every minute, someone will die of one (Lloyd-Jones *et al.*, 2010). Following cardiovascular stress, a cascade of compensatory structural events occurs within the myocardium and contributes to eventual left ventricular dysfunction and the manifestation of heart failure syndrome. MMPs have been extensively studied in various cardiac pathologies such as ischemia-reperfusion (I/R), dilated cardiomyopathy (DCM) and myocardial infarct (MI) (Janssens and Lijnen, 2006; Spinale, 2007; Brauer, 2006; Creemers *et al.*, 2001). Myocardial ischemia either with or without reperfusion induces ROS and pro-inflammatory cytokines. These reactive molecules are cardiodepressant through impairment of Ca^{2+} homeostasis. ROS can induce intracellular Ca^{2+} overload during oxidative stress. In addition, ROS directly injure the cell membrane and disturb the integrity of proteins, lipids and DNA, causing cell death (Hori and Nishida, 2009). However, low concentrations of specific ROS, such as peroxynitrite, may directly activate the zymogen forms of MMPs, as detailed in 1.2. In addition, MMPs may be activated by caspases after myocardial infarction (*cf.* 1.1).

During the last decade, Schulz and colleagues have accumulated a considerable amount of evidence on the intracellular localization and acute actions of MMPs, particularly MMP-2, in the heart. In contrast with most researchers, who have focused on the long term proteolytic effects of MMPs on ECM remodeling, they showed that MMP-2 contributes to acute cardiac mechanical dysfunction before the development of changes in the collagen matrix (Chow *et al.*, 2007b; Kandasamy *et al.*, 2010; Ali and Schulz, 2009; Schulz, 2007). Indeed, already after 20 minutes of global no-flow ischemia in the rat, a marked increase in pro-MMP-2 was observed in the coronary effluent that peaked within the first minute of reperfusion. The levels of pro-MMP-2 correlated positively with the duration of ischemia and negatively with the recovery of mechanical function. Contractile dysfunction associated with the induced I/R improved with a neutralizing MMP-2 antibody and with general MMP inhibitors (doxycycline and 1,10-phenantroline (O-phen)) (Cheung *et al.*, 2000).

Heart failure is characterized by a general decline in pump function caused by a decline in contractile properties of the cardiac myocytes. Myocardial contraction is initiated upon the release of Ca^{2+} into the cytosol from the sarcoplasmic reticulum following membrane depolarization. Thin filament activation and relaxation dynamics have emerged as a pivotal regulatory system tuning myofilament function to the

beat-to-beat regulation of cardiac output. Perturbation of thin filament dynamics is now recognized as an important cellular mechanism causing reduced cardiac pump function in a variety of cardiac diseases (Kobayashi *et al.*, 2008). I/R injury in the heart is associated with degradation or loss of myofilament regulatory proteins as well as structural and cytoskeletal proteins (Hein *et al.*, 1995; Matsumura *et al.*, 1996).

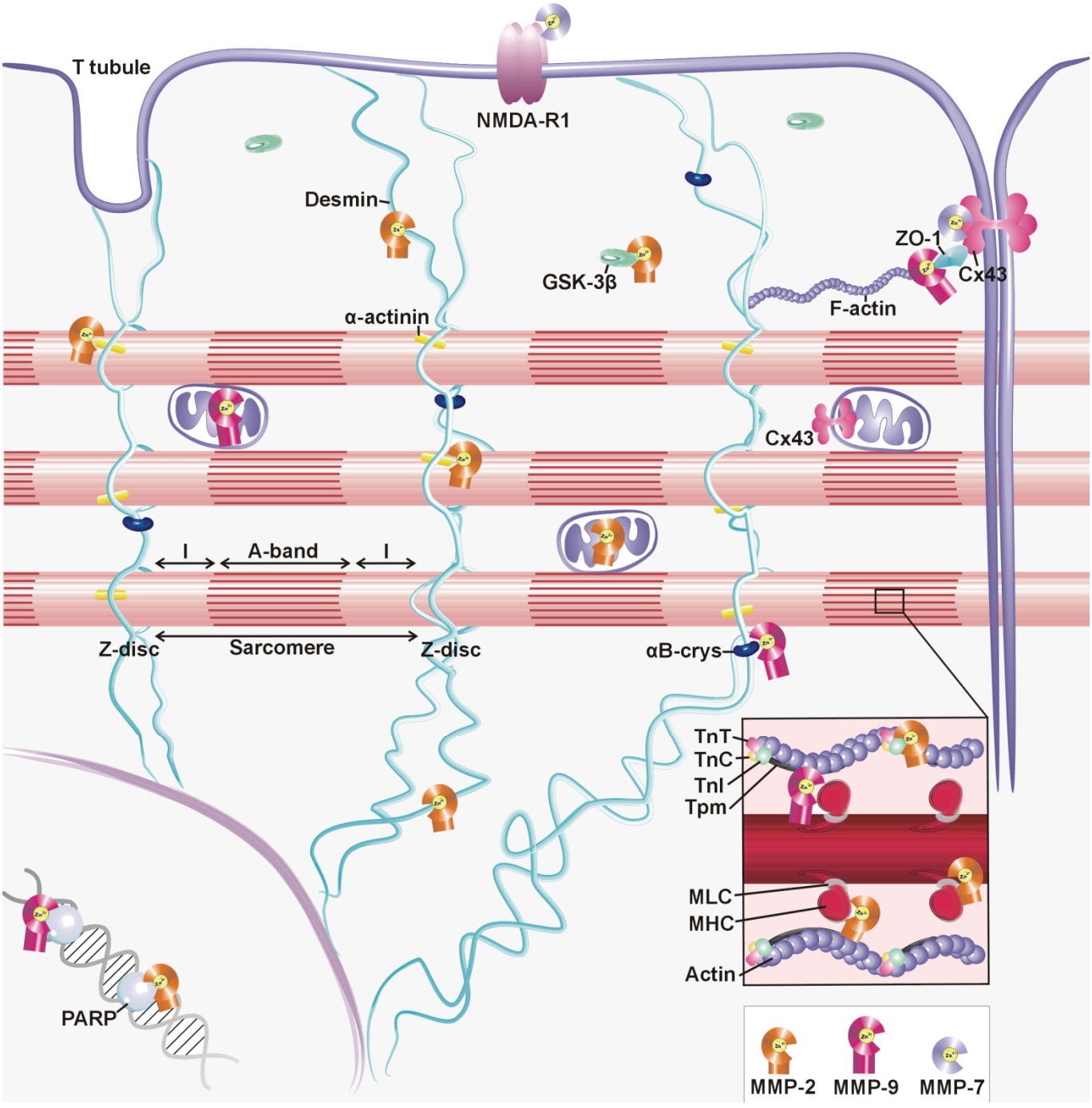

Figure 5. Intracellular proteolysis by MMPs in cardiac disease. MMPs may cause direct contractile dysfunction by the proteolysis of major sarcomeric proteins such as troponin I (TnI), myosin light chain (MLC) and myosin heavy chain (MHC). Intracardiomyocyte proteolysis by MMPs in cardiac injury also results in loss of cytoskeletal proteins, including α-actinin and desmin, and its chaperone, αB-crystallin (αB-crys). Proteolysis of connexin-43 (Cx43) leads to adverse electrical changes, probably by perturbation of the Cx43/zona occludens-1 (ZO-1) interaction. Under nitrosative stress, Cx43 translocates to the mitochondria, where its degradation leads to mitophagy and cell death, which may be ameliorated by cleavage of the *N*-methyl-*D*-aspartate receptor-1 (NMDA-R1). Proteolysis of glycogen synthase kinase-3β (GSK-3β) enhances its pro-apoptotic capacities and degradation of PARP in the nucleus also leads to enhanced apoptosis and cardiomyocyte death.

By immunogold electron microscopy, confocal microscopy, immunoprecipitation experiments and zymographic analysis of highly purified thin myofilament preparations, MMP-2 was localized to sarcomeres in close association with the thin myofilaments in hearts subjected to I/R (Wang *et al.*, 2002b). The thin filament is composed of F-actin together with two tropomyosin strands, each binding a troponin (Tn) complex (Figure 5). Tropomyosin and the Tn complex, composed of three subunits: troponin C (TnC) , I (TnI) and T (TnT), regulate the affinity of F-actin towards myosin. In resting conditions (absence of Ca^{2+}), TnI inhibits myosin cross-bridge formation to actin, and concomitant generation of myocardial force, via interactions with TnT and tropomyosin (Kobayashi *et al.*, 2008). Activated MMP-2 was found to co-localize with TnI (troponin I) and to rapidly degrade TnI *in vitro*. Inhibition of MMP-2 activity with doxycycline and O-phen prevented I/R-induced TnI degradation and improved the recovery of the mechanical function of isolated rat hearts. Importantly, no significant myocardial necrosis was observed, as TnI and its degradation products were not found in the coronary effluent, an additional proof of the intracellular localization of MMP-2 action (Wang *et al.*, 2002b). This was confirmed in another model of myocardial contractile failure, in which the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interferon-γ (IFN-γ) depressed myocardial contractile function by enhancing peroxynitrite production. MMP-2 activity was increased before the decline in myocardial mechanical function and this was followed by decreased levels of TnI in the cytokine-treated hearts, which was prevented by a neutralizing anti-MMP-2 antibody (Gao *et al.*, 2003). However, these neutralization experiments imply that the anti-MMP-2 antibody enters the intracellular milieu, for example by endocytosis, or that the binding and neutralization of MMP-2 occurs extracellularly and impedes the entry of MMP-2 in the myocyte.

Using 2D-PAGE, Schulz and colleagues found that myosin light chain (MLC-1) was degraded in hearts subjected to I/R injury and that this proteolytic process was inhibited with MMP inhibitors (Doxycycline and O-phen). Activated forms of MMP-2 were found in rat heart preparations of thick myofilaments, and by immunogold microscopy analysis, MMP-2 was localized in the sarcomere in a pattern consistent with the known distribution of MLC-1 (*cf.* Figure 5). In addition, purified MLC-1 was susceptible to MMP-2 (but not MMP-9) proteolysis in *vitro* (Sawicki *et al.*, 2005). Of interest, a different localization of the two gelatinases, MMP-2 and MMP-9, was observed in hearts of patients with dilated cardiomyopathy (DCM) compared to control hearts. In DCM hearts the gelatinases were localized exclusively within the cardiomyocytes in close association with the sarcomeric structure, whereas localization was mainly around the myocytes in control hearts. MMP-9, and to a lesser extent, MMP-2, cleaved purified myosin heavy chain (MHC) *in vitro* (*cf.* Figure 5) (Rouet-Benzineb *et al.*, 1999). Hence, MMPs may cleave both regulatory and structural elements that generate myocardial force, leading to mechanical dysfunction and heart failure.

This phenomenon may be enhanced by an imbalance between MMPs and TIMPs during cardiac injury. Indeed, during I/R in isolated rat hearts, TIMP-4 - the most abundant TIMP in the heart - was also rapidly released in the coronary effluent, as shown by reverse zymography and Western blot analysis. By immunogold microscopy analysis a close association of TIMP-4 with sarcomeres was demonstrated in aerobically perfused rat hearts, whereas this pattern of positive staining was reduced in I/R hearts. Although both MMP-2 and TIMP-4 were released into the extracellular milieu during reperfusion, a net proteolytic balance in hearts exposed to I/R was shown by *in situ* zymography of heart sections. These data suggest that TIMP-4 plays an important homeostatic role in the normal myocardium and that its release from the cardiomyocytes contributes to myocardial I/R injury (Schulze *et al.*, 2003). Likewise, all TIMPs were downregulated in DCM hearts, especially TIMP-1 (Rouet-Benzineb *et al.*, 1999), further pointing to the participation of MMP/TIMP imbalances in cardiac diseases.

As mentioned before, I/R injury is also associated with the degradation of cytoskeletal proteins such as α-actinin, desmin and spectrin (Matsumura *et al.*, 1996). This may constitute an additional intracellular function of MMP-2, as α-actinin and desmin (but not spectrin) were found to be *in vitro* substrates of MMP-2 (*cf.* Figure 5). α-actinin (α-actinin cytoskeletal isoform, non-muscle α-actinin-1, F-actin cross-linking protein) is an actin-binding protein. It forms an anti-parallel rodshaped dimer with one actin-binding domain at each end of the rod and bundles actin filaments in multiple cytoskeletal frameworks. Besides binding to actin filaments, α-actinin associates with a number of cytoskeletal and signaling molecules, for instance, cytoplasmic domains of transmembrane receptors and ion channels. Thus, α-actinin exerts major structural and regulatory roles in cytoskeletal organization and muscle contraction (Sjoblom *et al.*, 2008). Desmin is the main intermediate filament protein expressed in cardiac, skeletal, and smooth muscle cells. It interacts with other proteins to form a continuous cytoskeletal network that maintains a spatial relationship between the contractile apparatus and other structural elements of the cell, thus providing maintenance of cellular integrity, force transmission, and mechanochemical signaling. Mutations in desmin and in αB-crystallin (αB-crys), a chaperone for desmin and another MMP substrate (*vide infra*), lead to desminopathy, a myofibrillar myopathy (Goldfarb and Dalakas, 2009).

Peroxynitrite infusion into isolated rat hearts caused a decline in mechanical function and activation of MMP-2 with concomitant loss of intact α-actinin (but not desmin), which was prevented by an MMP inhibitor (PD-166793) or the peroxynitrite scavenger reduced glutathione (GSH). In addition, MMP-2 was found to co-localize with α-actinin in cardiomyocytes. Hence, these results suggest yet another contribution of MMP-2 to cardiac dysfunction by mediating cytoskeletal disorganization (Sung *et al.*, 2007).

In the heart, electrical stimulation readily spreads via direct cell-to-cell communication mediated by low resistance gap junction channels composed of proteins termed connexins, such that all myocytes contract in an ultra-fast wave at each beat. Hence, connexin channels ensure propagation of molecular and electrical signals and are crucial in myocardial synchronization and heart function (Tribulova *et al.*, 2008). Induction of myocardial infarct (MI) in MMP-7 knockout mice resulted in increased early survival and favorable alterations in electrical conduction patterns compared with wildtype mice. In wildtype mice, slower conduction velocity correlated with a 53% reduction in the gap junction protein connexin-43 (Cx43, gap junction 43 kDa heart protein, gap junction α-1 protein), which is the predominant connexin isoform in atrial as well as ventricular tissues. Because myocardial conduction patterns were altered, along with changes in gap junction proteins, the contributing factor for differences in survival was likely an alteration in electrical conduction due to MMP-7 genetic deletion. Surface plasmon resonance experiments revealed that MMP-7 binds to Cx43, and further *in vitro* analyses determined that MMP-7 cleaves Cx43 in the cytoplasmic COOH-terminal tail (*cf.* Figure 5). Infusion of MMP-7 induced Cx43 degradation and resulted in arrhythmias and heart block *in vivo* (Lindsey *et al.*, 2006). COOH-terminal truncation of Cx43 will abolish interaction with other proteins, such as zona occludens-1 (ZO-1), which was reported to have striking effects on gap junction size and distribution (Hunter *et al.*, 2005) and may contribute to the negative influence of MMP-7 on electrical conduction. Of interest, ZO-1 is an MMP-9 substrate (*vide infra*), which suggests that both MMPs may synergize in the perturbation of the Cx43/ZO-1 interaction, leading to adverse electrical changes and decreased survival after MI.

The mitochondria constitute yet another subcellular location wherefrom MMPs may disturb cardiac contractility. Both MMP-2 (Wang *et al.*, 2002b; Kwan *et al.*, 2004) and MMP-9 (Moshal *et al.*, 2008) are found in cardiac mitochondria. Elevated levels of blood homocysteine (HCY) (termed hyperhomocysteinemia) induced expression of MMP-9 in the mitochondrial compartment and induced mitochondrial permeability transition and contractile dysfunction, which was reversed by various compounds, including an inhibitor of the cardiomyocyte *N*-methyl-*D*-aspartate receptor-1 (NMDA-R1) (MK-801), a general MMP inhibitor (GM6001) and cyclosporine A. The intramitochondrial nitrosative stress induced by HCY in cardiomyocytes was associated with an increased translocation of Cx43 to the mitochondria and degradation of Cx43, and led to 'mitophagy' (mitochondrial autophagy) and cell death. This suggests that MMP-9 or another MMP may degrade Cx43 in the mitochondria and cause contractile dysfunction in hyperhomocysteinemia. These effects were ameliorated by cardiac-specific deletion of NMDA-R1 (Tyagi *et al.*, 2010). Interestingly, MMP-7 cleaves neuronal NMDA-R1 in the extracellular ligand-binding domain and this impairs NMDA-stimulated Ca^{2+} flux (Szklarczyk *et al.*, 2008). Hence, extracellular action of MMP-7 may be cardioprotective, whereas intracardiomyocyte cleavage of Cx43 by MMP-7 may cause heart failure, complicating the use of MMP inhibitors in cardiac disease.

In conditions of stress, cardiomyocytes mount an adaptive response that attempts to normalize ventricular wall stress and maintain cardiac output. Prolonged stress overwhelms this protective response and leads to cardiomyocyte apoptosis and heart failure. Glycogen synthase kinase-3β (GSK-3β) is a multifunctional Ser/Thr kinase that plays important roles in necrosis and apoptosis of cardiomyocytes. A major mechanism of cell necrosis is the opening of the mitochondrial permeability transition pore (mPTP). The threshold for mPTP opening is elevated by phosphorylation of GSK-3β at Ser9, which reduces activity of this kinase. In addition, inhibition of GSK-3β suppresses ATP hydrolysis by reducing ATP transport from the cytosol to the mitochondria. This prevents both ATP depletion and accumulation of inorganic phosphate, two factors promoting mPTP opening. Although the role of GSK-3β in apoptosis of cardiomyocytes has not been fully clarified, evidence to date supports its significant contribution to apoptosis induced by I/R, hypoxia/re-oxygenation, β-adrenoreceptor activation and pressure overload (Miura and Miki, 2009). GSK-3β was cleaved upon incubation with MMP-2 and this proteolytic cleavage of GSK-3β increased its activity. H_2O_2 challenge of H9c2 cardiomyoblasts increased the activity and level of MMP-2, reduced the level of GSK-3β and increased GSK-3β kinase activity. Both effects on GSK-3β were reduced by MMP inhibitors (GM6001 and ONO-4187). In addition, MMP-2 pull-down assays from H9c2 cell lysates showed the binding of MMP-2 with GSK-3β (Kandasamy and Schulz, 2009). Since inhibition of GSK-3β is cardioprotective for many reasons (Miura and Miki, 2009), augmentation of its activity may be an additional way of MMP-2 to contribute to cardiac injury resulting from enhanced oxidative stress. Interestingly, MMP-2 cleaves another

molecule that causes ATP depletion during oxidative stress, i.e. nuclear poly(ADP-ribose)polymerase (PARP, NAD^+ ADP-ribosyltransferase 1, poly(ADP-ribose) synthetase 1). PARP is a DNA repair enzyme. It is activated by DNA strand breaks, which may be caused by oxidative stress (Ahmad *et al.*, 2009). Both MMP-2 and MMP-9 were found in nuclear extracts of cardiomyocytes and hepatocytes (*cf.* Figure 5). MMP-2 was immunoprecipitated from nuclear extracts of heart cells with anti-PARP antibody and degraded PARP *in vitro* (Kwan *et al.*, 2004). Inhibitors of PARP have been shown to have a protective effect in cardiac injury (Pacher *et al.*, 2005). Mild damage to DNA activates the DNA repair machinery. In contrast, once excessive oxidative and nitrosative stress-induced DNA damage occurs, as in various forms of myocardial reperfusion injury and heart failure, overactivated PARP initiates an energy-consuming cycle by transferring ADP-ribose units from NAD^+ to nuclear proteins, resulting in rapid depletion of the intracellular NAD^+ and ATP pools, slowing the rate of glycolysis and mitochondrial oxidative phosphorylation (the generation of ATP), and eventually leading to cellular dysfunction and death. In addition, PARP also regulates the expression of a variety of pro-inflammatory mediators, which might facilitate the progression to heart failure. Hence, Schulz and colleagues suggest that nuclear MMP-2 may play a protective role during oxidative stress by proteolytic removal of the activated PARP overload (Schulz, 2007). This means that besides contrasting extracellular vs. intracellular functions (*cf.* MMP-7, *vide supra*), MMPs may also have opposed roles depending on their subcellular location.

In conclusion, MMP activities are detected in all subcellular compartments of the cardiomyocyte (*cf.* Figure 5) and generally contribute to dysfunction of the contractile apparatus and adverse electrical conduction after cardiac injury. Therapeutic intervention by MMP inhibition thus seems promising. However, as some subcellular MMP functions may be cardioprotective, adverse effects may be inevitable without the use of subcellular compartment-specific inhibitors.

3.3.4 Intracellular proteolysis in acute and chronic neurodegenerative diseases

Within seconds to minutes after the loss of blood flow to a region of the brain, the ischemic cascade is rapidly initiated and comprises a series of successive biochemical events. Hypoperfusion of a brain area leads to oxidative damage and excitotoxicity, which is the process by which neurons are damaged and killed *via* the overactivation of receptors for the excitatory neurotransmitter glutamate, such as the *N*-methyl-*D*-aspartate receptor-1 (NMDA-R1) and the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA-R) receptor. Oxidative stress and excitotoxicity in turn cause microvascular injury, blood-brain barrier (BBB) dysfunction and post-ischemic inflammation, ultimately resulting in cell death of neurons, glia and endothelial cells. These events exacerbate the initial injury and may lead to permanent cerebral damage, the extent of which is dependent on the degree and duration of ischemia (Lakhan *et al.*, 2009; Lee *et al.*, 2004b). The extracellular participation of MMPs in cerebral ischemia has been well documented. MMPs cause the increase in permeability of the BBB by attacking the endothelial cell tight junctions (TJs) and the basal lamina's, i.e. the subendothelial and the parenchymal basement membranes (Agrawal *et al.*, 2006), resulting in the final common pathway downstream of acute neuroinflammatory damage. When acute hypoxia–ischemia initiates the cellular damage, MMPs target the matrix proteins around blood vessels and brain cells, resulting in cytotoxic and vasogenic edema, hemorrhagic transformation, and apoptosis of neurons and oligodendrocytes (Candelario-Jalil *et al.*, 2009; Rosenberg, 2009; Cauwe *et al.*, 2007).

The BBB is a diffusion barrier, consisting of an interdependent network of cells designed to segregate the central nervous system (CNS) from the systemic circulation. One of the primary functions of the BBB is the strict regulation of paracellular permeability, which is mediated by the endothelial tight junctions (TJs) of the capillary that limit paracellular movement of solutes, ions, and water. The preservation of the TJs is governed by three essential transmembrane proteins: claudins, occludin, and junction adhesion molecules (*cf.* Figure 6). The cytoplasmic regions of these transmembrane proteins are attached to intracellular scaffolding proteins, such as zona occludens-1,-2 and -3 and cingulin, which in turn are anchored to the actin cytoskeleton. Treatment of brain endothelial cells with activated MMP-9 resulted in an increased frequency of discontinuities in the immunohistological staining of zona occludens-1 (ZO-1, tight junction protein 1) and a consistent decrease in the intensity of ZO-1 expression (Harkness *et al.*, 2000). ZO-1 was degraded *in vivo* during transient and permanent focal ischemia and this was attenuated with MMP inhibitors (BB-94 and KB-R7785) (Jiang *et al.*, 2001b; Asahi *et al.*, 2000; Bauer *et al.*, 2010b) and in MMP-9 knockout mice, with concomitant reduction of BBB disruption and infarct size (Asahi *et al.*, 2001). Degradation of ZO-1 was also observed after treatment of rat brain endothelial cells with activated MMP-13 (Lu *et al.*, 2009). However, since ZO-1 is a cytoplasmic protein, degradation must occur intracellularly by cytoplasmic proteases. Indeed, cerebral ischemia and hypoxia increased both MMP-9 expression and activation in brain endothelial cells (Bauer *et al.*, 2010a; Asahi *et al.*, 2000), suggesting that endogenous intracellular MMP-9 cleaves ZO-1. However, hypoxic conditioned medium of astrocytes (with upregulated levels of

MMP-2, -9 and -13) also induced ZO-1 cleavage in brain endothelial cells, which was reversed with neutralizing antibodies against MMP-9 and MMP-13. Hence, alternative explanations are that extracellular MMPs trigger ZO-1 cleavage indirectly, or that MMPs are internalized, for example by endocytic mechanisms as discussed in 3.2.1.

The aberrant, excessive activity of extracellular MMPs contributes directly to neuronal cell death in multiple ways, including modulation of anoikis (Gu *et al.*, 2002) and calpain activity (Copin *et al.*, 2005). Of interest, S-nitrosylated MMP-9 (*cf.* 1.2) may not only induce neuronal apoptosis by anoikis, but possibly also by the cleavage of actin. Indeed, increased pro- and activated MMP-9 levels in ischemic brains were associated with slightly decreased levels of actin (Gu *et al.*, 2002), and cleavage of actin and other cytoskeletal proteins is pro-apoptotic (*vide infra*). In addition, MMPs may also contribute to neuronal apoptosis by an intranuclear function. Indeed, various studies show early upregulation of gelatinolytic activity in the nucleus after an ischemic insult (Gasche *et al.*, 2001; Amantea *et al.*, 2008; Gu *et al.*, 2005; Yang *et al.*, 2007). The MMPs identified in the nucleus were MMP-2, MMP-9, MMP-13 and MT1-MMP (*cf.* Figure 6) (Yang *et al.*, 2010; Cuadrado *et al.*, 2009). In addition, furin was found co-localized with MT1-MMP and MMP-2 in ischemic cell nuclei, which may trigger the MMP activation cascade (*cf.* 1.1). MMP inhibition (CH6631) reduced cerebral infarction as well as cerebral ischemia-induced apoptosis, as evidenced by reduced DNA fragmentation and cytochrome c release and increased intact PARP (Copin *et al.*, 2005). Purified PARP was cleaved *in vitro* by MMP-2 and MMP-9, and by less defined gelatinase preparations and total nuclear extracts from ischemic brains. In addition, this cleavage was inhibited by an MMP-2/-9 inhibitor. PARP activity was significantly reduced in ischemic brains compared with those treated with BB1101. Activated PARP recruits X-ray cross-complementary factor 1 (DNA repair protein XRCC1) which is also cleaved by MMP-2 and MMP-9 in *vitro*, as well as by gelatinase preparations and total nuclear extracts. Nuclear gelatinolytic activity co-localized with PARP and XRCC1 staining in ischemic brains and their *in vivo* degradation during ischemia was reduced by the MMP inhibitor BB1101. In addition, accumulation of oxidative DNA damage was also reduced by BB1101 (Yang *et al.*, 2010). As discussed above (*cf.* 3.3.3), PARP is activated by DNA strand breaks caused by oxidative stress. XRCC1 is a substrate of PARP and is recruited by PARP to sites of DNA damage. XRCC1 functions as a scaffold protein able to coordinate and facilitate the steps of various DNA repair pathways by interacting with major DNA repair enzymes (Horton *et al.*, 2008). In response to cellular damage by oxygen radicals or excitotoxicity, a rapid and strong activation of PARP occurs in neurons and excessive PARP activation has been implicated in cerebral ischemia (Skaper, 2003). Hence, Yang and colleagues suggest that in mild damage of early stage I/R injury, proteolysis of PARP1 and XRCC1 may contribute to oxidative DNA damage and neuronal apoptosis by impairing their protective action in the DNA repair pathway. However, in later disease stages, intranuclear MMP activity may contribute to recovery by clearing the burden of over-activated PARP1 that causes neuronal necrosis (Yang *et al.*, 2010). Hence, the beneficial effects of MMP inhibitors in the early stages would need to be balanced with later interference in recovery. This scheme also implies specific time windows for the treatment of brain ischemia with MMP inhibitors (Hu *et al.*, 2007).

Intracellular proteolysis during brain inflammation not only affects neuronal survival but may also adversely influence synaptic function. Application of recombinant MMP-7 to cultured rat neurons induced long-lasting inhibition of vesicular recycling as well as reduced local abundance of vesicular and active zone proteins within synaptic terminals. Chronic application of MMP-7 resulted in synaptic atrophy, including smaller terminals and fewer synaptic vesicles (Szklarczyk *et al.*, 2007a). On the one hand, this effect may be explained by extracellular proteolysis of ECM, adhesion molecules and receptors such as NMDAR-1 (Szklarczyk *et al.*, 2008). On the other hand, MMP-7 was evidenced to proteolyse the presynaptic protein synaptosomal-associated protein of 25 kDa (SNAP-25) (*cf.* Figure 6). SNAP-25 is a cytosolic soluble N-sensitive factor attachment protein receptor (SNARE) complex protein that participates in the regulation of synaptic vesicle exocytosis (Matteoli *et al.*, 2009). MMP-7 generates a long-lasting (7 days) 15 kDa SNAP-25 fragment by cleavage at the Ala128-Ile129 peptide bond (Szklarczyk *et al.*, 2007b). MMP-7-mediated proteolysis of SNAP-25 was inhibited by inhibitors of clathrin-dependent endocytosis (*cf.* 3.2.1). MMP-7 may thus share a particular function with clostridial toxins, which enter neuronal cells *via* receptor-mediated endocytosis and cleave SNARE proteins, generating long-lasting fragments and perturbing neurotransmitter exocytosis (Bajohrs *et al.*, 2004). Hence, exogenous MMP-7 is able to access and cleave an intraneuronal substrate and may impair neurotransmission during brain inflammation.

Besides their roles in neuronal apoptosis after acute cerebral I/R injury, intranuclear MMPs may also affect chronic neurodegenerative diseases such as Parkinson's disease (PD). PD results from neurodegeneration of dopaminergic neurons in the *substantia nigra*, which is associated with activation of microglia and accumulation of aggregated α-synuclein in specific brain stem, spinal cord, and cortical regions (Lees *et al.*, 2009). Recent studies have implicated MMPs in the death of dopaminergic neurons in this disease.

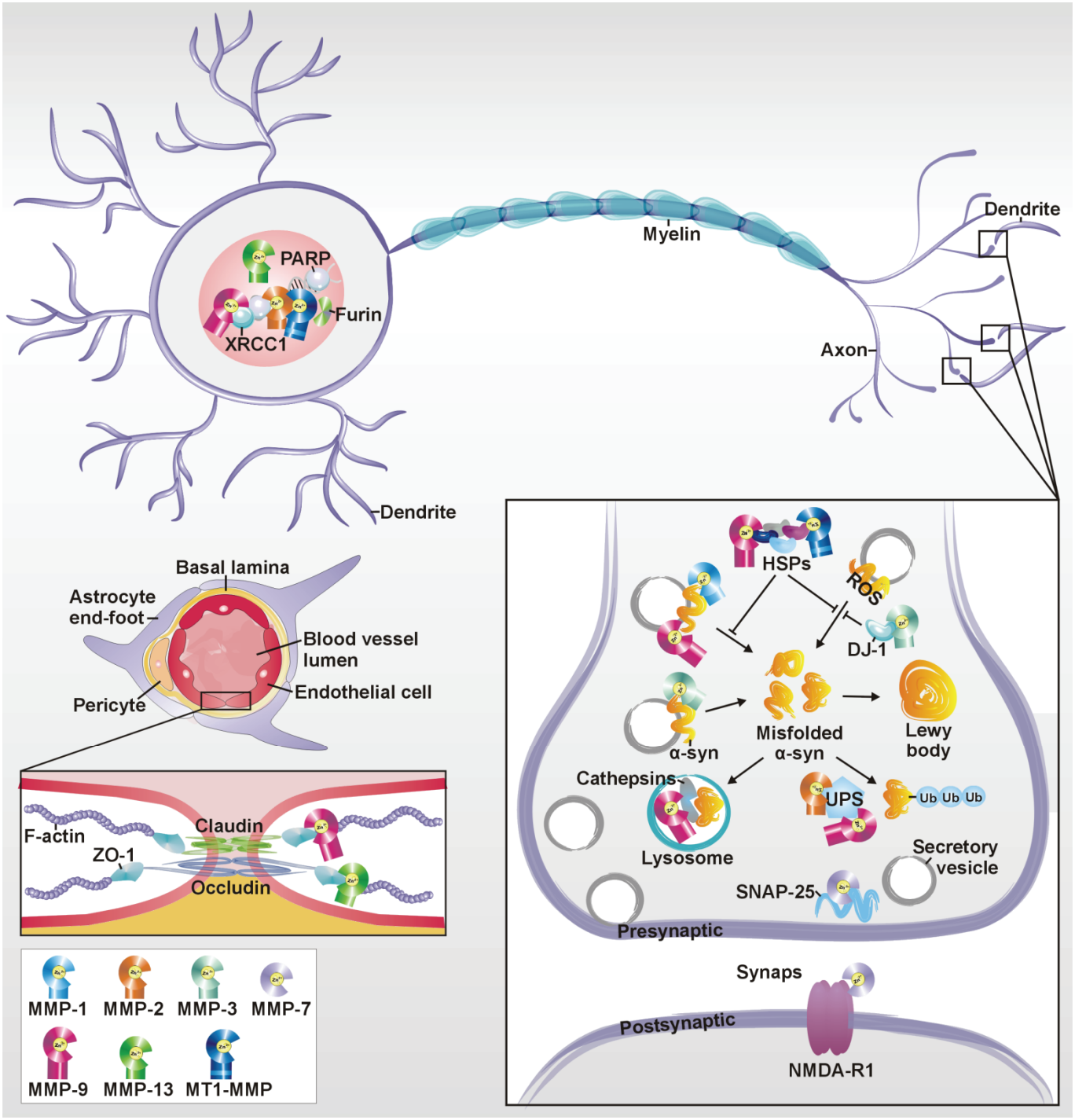

Figure 6. Intracellular proteolysis by MMPs in acute and chronic neurodegeneration. In acute cerebral ischemia, MMPs contribute to blood brain barrier disruption by the extracellular degradation of claudin and occludin, and by the intracellular proteolysis of zona occludens-1 (ZO-1). Furthermore, activated MMP-2 and MMP-9 in the nucleus may cleave poly (ADP-ribose) polymerase (PARP) and X-ray cross-complementary factor 1 (XRCC1), which causes apoptosis and neuronal cell death early after ischemia as it impairs DNA repair after oxidative DNA damage. However, in later disease stages the clearance of the overactivated PARP overload may contribute to recovery. MMP-2 may be activated by MT1-MMP, which in turn may be activated by furin, since all three proteases were found to colocalize in the nuclei of ischemic neurons. In presynaptic terminals, proteolysis of synaptosomal-associated protein of 25 kDa (SNAP-25) may perturb synaptic vesicle exocytosis during neuroinflammation. Intracellular proteolysis by MMPs also has pathogenic effects in chronic neurodegenerative diseases, such as Parkinson's disease. MMPs cleave α-synuclein and increase its aggregation, which may lead to enhanced accumulation of toxic Lewy bodies. By degrading protective chaperones (e.g. heat shock proteins) and anti-oxidant molecules (e.g. DJ-1), MMPs also contribute indirectly to toxic protein precipitation and aggregation. Finally, MMPs may also perturb the clearance of misfolded proteins by inactivating many proteins of the ubiquitin-proteasomal system (UPS) and lysosomal proteins.

In vitro, apoptotic dopaminergic neurons under cellular stress released activated MMP-3, but not the pro-form, which acted as a microglia activating molecule. Both released activated MMP-3 and the catalytical domain of MMP-3 led to the production of superoxide and microglial inflammatory cytokines such as TNF-α, which in turn exacerbated neuronal apoptosis and necrosis. In the N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-injected animal model of PD, neuronal degeneration, microglial activation, and superoxide generation were largely attenuated in MMP-3 knockout mice (Kim *et al.*, 2005; Kim *et al.*, 2007). An activated form of MMP-3 was generated inside stressed dopaminergic cells by a serine protease different from furin (*cf.* 1.1). This intracellular activated form of MMP-3 (but not the extracellular MMP-3) was found to participate in apoptotic signaling, upstream of caspase-3. Hence, these results suggest that MMP-3 catalytic activity seems to play different synergistic roles in dopaminergic neuron degeneration, both intracellularly by promoting neuronal apoptosis and extracellularly by triggering neuroinflammation (Choi *et al.*, 2008).

In PD, dopaminergic neurons of the *substantia nigra* show toxic intracellular filamentous inclusions termed Lewy bodies, which consist mainly of an abnormal, post-translationally modified, and aggregated form of α-synuclein (α-syn, non-Aβ component of AD amyloid, non-A4 component of amyloid precursor, NACP) (*cf.* Figure 6). α-syn is a cytoplasmic (presynaptic) protein that normally regulates neurotransmitter release, possibly *via* interaction with lipid domains on secretory vesicles (Sulzer, 2010). Of interest, MMP-3 and MMP-9, but not MMP-2, were found to co-localize with α-syn in Lewy bodies (Personal communications from Johannes Levin and Stefan Lorenzl, University of Munich, Germany and from Yoon-Seong Kim, Weill Cornell Medical College, New York). Moreover, dopaminergic neuroblastoma cells under oxidative stress showed an upregulation of intracellular and secreted activated forms of MMP-3 and cleavage of α-syn, which was inhibited by an MMP inhibitor (MMP inhibitor II). Purified α-synuclein is cleaved by MMP-3 most efficiently, but also by MT1-MMP, MMP-2, MMP-1 and MMP-9 (ordered by decreasing efficiency) (Sung *et al.*, 2005). Many non-overlapping cleavage sites were found for the cleavage of α-syn by MMP-3, -1 and -9 (*cf.* Table 3) (Levin *et al.*, 2009). The tested MMPs cleaved preferentially in the COOH-terminal part of α-syn in and around the non-Aβ component of Alzheimer plaques (NAC) domain. The NAC domain is a hydrophobic region that is highly prone to aggregation. Indeed, limited proteolysis by MMP-1 and MMP-3, but not by MMP-9, increased α-syn aggregation. In addition, α-syn fragments produced by MMP-3 facilitated aggregation of intact α-syn and had a more toxic effect on cell viability. However, high MMP concentrations, which resulted in pronounced degradation of α-syn, blocked subsequent aggregation. Hence, it seems that, under oxidative stress, MMPs, especially MMP-3, may regulate α-syn aggregation and toxicity in dopaminergic neurons, thereby affecting onset and progression of neurodegeneration in PD. In addition, MMP-3 was suggested to cleave protein DJ-1 (DJ-1, oncogene DJ1, Parkinson disease protein 7) (personal communication from Yoon-Seong Kim), and various degradomics screens identified DJ-1 as an *in vitro* substrate of MMP-1,-2,-8,-9 and MT1-MMP (*cf.* Table 2) (Dean *et al.*, 2007; Dean and Overall, 2007; Butler *et al.*, 2008). DJ-1 is an important redox-reactive signaling intermediate controlling oxidative stress after ischemia, upon neuroinflammation, and during age-related neurodegenerative processes such as PD (Kahle *et al.*, 2009). In conclusion, inhibition of these pathogenic actions of MMP(-3) may have a neuroprotective effect, as was already shown *in vitro* and *in vivo* with doxycycline in the MPTP animal model of PD (Cho *et al.*, 2009).

Besides causing direct aggregation of substrates by proteolysis, MMPs may also contribute indirectly to protein aggregation in neurodegenerative diseases such as PD by the inactivation of protective proteins such as chaperones, proteins from the ubiquitin- proteasomal system (UPS) and proteins that function in lysosomal degradation. Indeed, heat shock proteins (HSPs) play a substantive role in PD pathology and chaperone therapy was recently proposed as a novel treatment strategy (Bandopadhyay and de, 2010; Luo and Le, 2010). Indeed, extensive localization of several HSPs with α-syn in Lewy bodies has been demonstrated, including the (candidate) MMP substrates HSP27, HSP70 and HSP90. HSPs inhibit α-syn toxicity and aggregation. This was shown for another small HSP, αB-crystallin, which has protective functions in the heart (*cf.* 3.3.3) and in multiple sclerosis brain (*cf.* 4.2.1) and is also an MMP substrate. In addition, heat shock cognate protein 70 (HSC70) and HSP90 facilitate the transfer of α-syn from the cytosol to the lysosomes for degradation (Bandopadhyay and de, 2010). Furthermore, failure of the UPS system has recently emerged as an additional pathogenic factor that underlies development of familial and sporadic PD (McNaught *et al.*, 2001). Hence, inactivation of UPS proteins by MMPs may contribute to the neurotoxic protein accumulation and aggregation observed when the UPS system fails. Finally, additional defects in protein clearance may be caused by MMP-mediated degradation of lysosomal proteins (*cf.* Table 3). In conclusion, inhibition of MMPs in PD may not only avoid α-syn truncation and aggregation, but may also preserve chaperone function, and maintain effective protein degradation and clearance.

Neuronal survival requires continuous lysosomal turnover of cellular constituents delivered by

autophagy and endocytosis. Hence, the lysosomal system is a convergence point for a surprising number of genetic mutations that cause neurodegenerative diseases (Nixon *et al.*, 2008). Of interest, various MMP (candidate) substrates are lysosomal proteins that contain mutations in neurodegenerative disorders, including cathepsins D and E, Niemann-Pick, type C2 (NPC2), proganulin and triose phosphate isomerase (*cf.* Table 5). Intracellular modification of these proteins by MMPs may constitute an unexplored pathogenic factor contributing to neurodegeneration in these rare monogenetic diseases. Likewise, other proteins with mutations causing storage diseases are (potential) targets of MMPs, such as enolase-β, aldolase A, β-glucuronidase, iduronate-2-sulfatase, and filamin C. This is certainly an unexpected but interesting link that needs further clinical research.

3.3.5 Intracellular proteolysis and cataract

The human lens is suspended in a complex environment. The front side is constantly rinsed by the aqueous fluid, while the gel-like vitreous acts as a shock breaker. The lens is composed of three main types of proteins, α-, β- and γ-crystallins. Crystallins focus light on the retina by maintaining the necessary refractive characteristics and transparancy of the lens. Besides this structural role, crystallins may also function as chaperones and suppress the aggregation of proteins denatured by oxidation, heat, and other stressors. Crystallins are constantly subjected to changes such as oxidation, deamidation, truncation, glycation, and methylation that accumulate with time. Such age-related modifications affect crystallin structure and function. With time, the modified crystallins aggregate, causing the lens to increasingly scatter light instead of focusing light on the retina, and causing the lens to gradually lose its transparency and become opaque. This age-related lens opacity, or cataract, is the major cause of blindness worldwide. Calpains have been implicated in the proteolytic modification of crystallins but their role in human cataractogenesis is yet to be established, primarily because of the presence of a calpain inhibitor in the lens at a several fold higher concentration than calpain itself (Sharma and Santhoshkumar, 2009). *Ex vivo* incubation of mouse lenses with recombinant activated MMP-9 was shown to cause opacification of the lens within 15 min (Descamps *et al.*, 2005). 2D-PAGE analysis of extracts from these lenses showed the disappearance of intact βB1-crystallin (βB1-crys) and the appearance of a βB1-crys fragment, as well as a minor βB3-crys fragment. Truncation of βB1-crys by MMP-9 was confirmed *in vitro* in crude eye extracts and the scissile bond was identified at Ala47-Lys48 by Edman degradation. *In vitro* degradation of eye extracts also identified a fragment of γC-crys. However, as this fragment was not found in the *ex vivo* opacificated lenses, it is not likely to be an *in vivo* target of MMP-9 in murine cataract. Injection into the vitreous chamber of activated MMP-9 or the mouse CXC chemokine, granulocyte chemotactic protein-2 (GCP-2), to attract neutrophils as an endogenous source of MMP-9, resulted in βB1-crys degradation *in vivo*, whereas such degradation was not observed in the lenses of MMP-9 knockout mice. *Ex vivo* development of cataract in the presence of activated MMP-9 was accompanied by enzymatic loosening of the lens capsule, which is an uninterrupted basement membrane enclosing the lens (Descamps *et al.*, 2005). Since the lens capsule is composed of the same molecules as most basement membranes (Danysh and Duncan, 2009), it is not surprising that MMP-9 or other MMPs may penetrate it and subsequently enter the lens fiber cells by an undefined mechanism. Of interest, αB-crys, a more prominent crystallin in the eye, is also cleaved by MMP-9 and other MMPs (*vide infra*) (Starckx *et al.*, 2003; Shiryaev *et al.*, 2009). Although extensive αB-crys fragmentation was observed in human aged lenses (Sharma and Santhoshkumar, 2009), it was not shown to be an MMP target in the above-mentioned mouse study. However, this may be accounted for by interspecies differences and the animal model used, and does not exclude αB-crys as an (MMP) target in human cataract.

In conclusion, lens crystallins were among the first intracellular MMP substrates discovered by *in vivo* studies with knockout mice and with direct pathological implications in cataract development.

3.3.6 Intracellular proteolysis and apoptosis

Extracellular MMPs may affect cellular apoptosis in multiple ways with both pro- and anti-apoptotic outcomes (Mannello *et al.*, 2005). Many effects of intracellular proteolysis by MMPs on cell death have been described in previous sections, such as the anti-apoptotic effect of mitochondrial MMP-1 (*cf.* 3.3.2) (Limb *et al.*, 2005) and cleavage of GSK-3β by MMP-2, which enhances its pro-apoptotic and other pathogenic activities in the heart (Kandasamy and Schulz, 2009). Intranuclear activities of MMP-9 and MMP-2 after cerebral ischemia may inactivate the nuclear matrix proteins PARP-1 and XRCC1, causing apoptosis and necrosis early after ischemia, but protecting neuronal cells from death at later stages in the evolution of ischemic lesions (Yang *et al.*, 2010). Activated MMP-3 inside stressed dopaminergic neurons contributes to pro-apoptotic signaling upstream of caspase-3 (Choi *et al.*, 2008). The active form of MMP-3 (but not the pro-form) was already mentioned to be efficiently transported to the nucleus by means of a nuclear localization signal (*cf.* 3.2.6). Cell populations expressing a nuclear activated form of MMP-3 (by transfection) contained higher percentages of apoptotic cells than the control populations transfected with pro-MMP-3 (remaining cytosolic), as

determined by immunofluorescent staining for activated caspase-3. This induction of apoptosis was dependent on the catalytic activity of MMP-3, as transfection of an inactive mutant or treatment with the broad spectrum MMP inhibitor GM6001 significantly reduced the apoptotic index. Hence, nuclear MMP-3 induces apoptosis by its proteolytic activity (Si-Tayeb *et al.*, 2006). In completely different settings, cigarette smoke-induced apoptosis was associated with gelatinolytic activity in the nucleus, increased pro- and activated MMP-2 levels in nuclear and cytosolic fractions and early PARP-1 fragmentation before the time point of caspase-3 activation, again suggesting a role for MMP-2 activity in nuclear matrix proteolysis during apoptosis (Ruta *et al.*, 2009). Of interest, MMP-2 expression and activation were reduced by inhibitors of two major apoptotic pathways in endothelial cells, caspase- and p38 mitogen-activated protein kinase (MAPK)-induced apoptosis, i.e. the pan caspase inhibitor Z-VAD and the p38 inhibitor SB203580. Curiously, Z-VAD increased, whereas SB203580 decreased MT1-MMP expression and activity. These results would suggest that alteration of MMP-2 activity is rather effect than cause in the apoptotic cascade. However, addition of pro- and activated MMP-2 to endothelial cell cultures showed a dose-dependent induction of apoptosis. Elevation of p38 and its phosphorylation were observed following the addition of active MMP-2, pointing to a positive regulatory loop for MMP-2 in the p38 MAPK apoptotic pathway. However, neither pro-MMP-2 nor activated MMP-2 influenced the levels of active caspase-3, suggesting that MMP-2 is downstream of caspases (Shapiro *et al.*, 2010). Indeed, a recent study by Yarbrough *et al.* showed increased MMP activity after treatment of heart homogenates with active caspase-3 and generation of activated forms of MMP-2 by an active caspase cocktail, suggesting direct proteolytic activation of MMPs by active caspases (as discussed in 1.1) (Yarbrough *et al.*, 2010).

If one analyzes critically the apoptotic cascade by evaluation of intracellular substrates, rather than by scrutinizing the enzymes, it becomes factual knowledge that most of the well-defined intracellular MMP substrates (*cf.* Table 5) are also cleaved by pro-apoptotic proteases such as calpains, caspases and granzymes, as summarized in Table 6. Strikingly, as can be deduced from Table 6, cleavage of these proteins by MMPs or (other) apoptotic proteases seems to have a general pro-apoptotic effect (Figure 7). In addition, from degradomics substrate analysis (Table 3), it is clear that other important apoptosis regulators may be modulated by MMPs. These regulators include BH3-interacting domain death antagonist (BID), apoptosis-linked-gene-2-interacting-protein X (ALIX or programmed cell death protein 6) and cytochrome c. Granzyme A induces apoptosis by multiple cleavages, including the cleavage of histones, a process that opens up chromatin to DNases, and proteolysis of high mobility group B2 (HMGB2), which liberates the DNase activity of nucleoside diphosphate kinase A (Lieberman and Fan, 2003). Both, histones and HMGB2, are high-confidence candidate substrates of MMPs (*cf.* Table 3).

As mentioned in chapter 2, MMPs seem to degrade a whole array of intracellular matrix (ICM) proteins. Cleavage and reorganization of cytoskeletal proteins is essential in apoptosis progression as it ensures the systematic dismantling of the dying cell and probably contributes to the early cell rounding and retraction, and partly to apoptotic membrane blebbing (Ndozangue-Touriguine *et al.*, 2008; Taylor *et al.*, 2008). Indeed, MMPs degrade many focal adhesion proteins, including FAK, α-actinin and the filamins (*cf.* Tables 3 and 5 and Figure 7). MMPs may also participate in the degradation of the actin meshwork surrounding the nuclear lamina, which disrupts the attachment between the actin cytoskeleton and the nuclear envelope, tearing the nucleus apart during apoptosis. Of interest, apoptosis in ischemic brains was associated with increased levels of pro- and S-nitrosylated active MMP-9 and decreased levels of actin (*cf.* 3.3.4) (Gu *et al.*, 2002). In addition, and again similarly to caspases, MMPs seem to target many proteins that are involved in essential housekeeping functions in the cell, such as regulation of transcription and translation, protein biosynthesis and carbohydrate metabolism (*cf.* Table 3). This is in line with the early shutdown of transcription and translation observed during apoptosis and may shut off the life-support systems of the cell (Taylor *et al.*, 2008). Furthermore, Tables 3 and 6 show that MMPs also target many heat shock proteins and other molecular chaperones, which are known to have multiple anti-apoptotic effects (Beere, 2004; Lanneau *et al.*, 2008). In conclusion, MMPs have many substrates in common with pro-apoptotic proteases and most MMP effects on substrates are pro-apoptotic (*cf.* Figure 7). The observation that exceptions to this rule exist, strengthens the fact that by such proteolysis a regulatory role is played in the apoptotic process, and this is in line with the well-known duality of pro- and anti-apoptotic roles of MMPs (Mannello *et al.*, 2005).

Both, the calpains and caspases, are families of cysteine proteases that have important roles in the initiation, regulation and execution of cell death (Harwood *et al.*, 2005). For these enzymes it is generally accepted that they act intracellularly. Of interest, Schulz and coworkers discovered that the general calpain inhibitor calpastatin inhibits MMP-2 *in vitro*. Since much of the evidence for calpain degradation of substrates is based on the use of calpain inhibitors, calpains may have been incorrectly identified as the proteases responsible for some intracellular proteolytic activities (Kandasamy *et al.*, 2010).

Table 6. Cleavage of intracellular MMP substrates by pro-apoptotic proteases and effect of substrate cleavage on the apoptotic pathway

Substrate*	MMP	Calpain	Caspase	granzyme	Effect of cleavage	References
INTERCELLULAR JUNCTION PROTEINS						
Cx43	MMP-7	ND	ND	ND	pro-apoptotic	(Giardina *et al.*, 2007)
ZO-1	MMP-9	ND	caspase X	ND	pro-apoptotic	(Bojarski *et al.*, 2004)
CYTOSKELETAL PROTEINS						
α-actinin	MMP-2	calpain X	capase-3	NC	pro-apoptotic	(Selliah *et al.*, 1996; Communal *et al.*, 2002; Nakamura *et al.*, 1993; Triplett and Pavalko, 2006)
CAP1	MMP-9 MMP-2,-8,-13	ND	ND	ND	anti-apoptotic	(Wang *et al.*, 2008)
Desmin	MMP-2	calpain 1 calpain 2	caspase-6	ND	pro-apoptotic	(Whipple and Koohmaraie, 1991; Chen *et al.*, 2003; Papp *et al.*, 2000)
FAK	MT1-MMP MT3-MMP	calpain 1 calpain 2	caspase-3,-7 capase-6,-8	granzyme B	pro-apoptotic	(Carragher *et al.*, 1999; Wen *et al.*, 1997; Gervais *et al.*, 1998; Cance and Golubovskaya, 2008)
Gelsolin	MMP-1,-2,-3,-7,-9	calpain X	caspase-3	ND	pro-apoptotic	(Fujita *et al.*, 1999; Geng *et al.*, 1998; Kothakota *et al.*, 1997; Wolf *et al.*, 1999)
Pericentrin	MT1-MMP	NC	NC	NC	pro-apoptotic	(Zimmerman *et al.*, 2004)
SARCOMERIC PROTEINS						
MLC-1	MMP-2	calpain 3	caspase-3	granzyme A	pro-apoptotic	(Cohen *et al.*, 2006; Moretti *et al.*, 2002; Nakamura *et al.*, 1993)
MHC	MMP-2 MMP-9	calpain 2	caspase X	granzyme A	pro-apoptotic	(Nakamura *et al.*, 1993; Gerner *et al.*, 2000; Azarian *et al.*, 1993)
TnI	MMP-2	calpain 1 calpain 2	NC	ND	pro-apoptotic	(Gao *et al.*, 1997; Ruetten *et al.*, 2001; Di Lisa *et al.*, 1995)
MOLECULAR CHAPERONES						
αB-crys	MMP-9	calpain 2	ND	ND	pro-apoptotic	(Kelley *et al.*, 1993; Kamradt *et al.*, 2001)
βB1-crys	MMP-9	calpain2 CSS2	ND	ND	ND	(Ma *et al.*, 2004; David *et al.*, 1993)
βB3-crys	MMP-9	calpain 2	ND	ND	ND	(David *et al.*, 1993)
γC-crys	MMP-9	calpain 2 calpain 3	ND	ND	ND	(Baruch *et al.*, 2001; Tang *et al.*, 2007)
PRESYNAPTIC PROTEINS						
α-syn	MMP-1,-2,-3,-9 MT1-MMP	calpain 1 calpain 2	ND	ND	anti-apoptotic	(Mishizen-Eberz *et al.*, 2003; Kim *et al.*, 2004)
SNAP-25	MMP-7	calpain 2	ND	ND	ND	(Ando *et al.*, 2005)
NUCLEAR MATRIX PROTEINS						
PARP-1	MMP-2	calpain 1	capase-1,-3	granzyme A granzyme B	pro-apoptotic	(Buki *et al.*, 1997; Tewari *et al.*, 1995; Gu *et al.*, 1995; Froelich *et al.*, 1996; Zhu *et al.*, 2009)
XRCC1	MMP-2 MMP-9	ND	ND	ND	pro-apoptotic	(Yang *et al.*, 2010)
ANTIBACTERIAL PEPTIDES						
pro-Crps	MMP-7	ND	ND	ND	ND	/
pro-CRS4C	MMP-7	ND	ND	ND	ND	/
SIGNALING PROTEINS						
ER-β	MMP-26	ND	ND	ND	anti-apoptotic	(Lazennec, 2006)
GSK-3β	MMP-2	calpain 1	ND	ND	pro-apoptotic	(Goni-Oliver *et al.*, 2007)

*The meaning of the acronyms can be found in Table 5.

NC, not cleaved; **ND**, not defined.

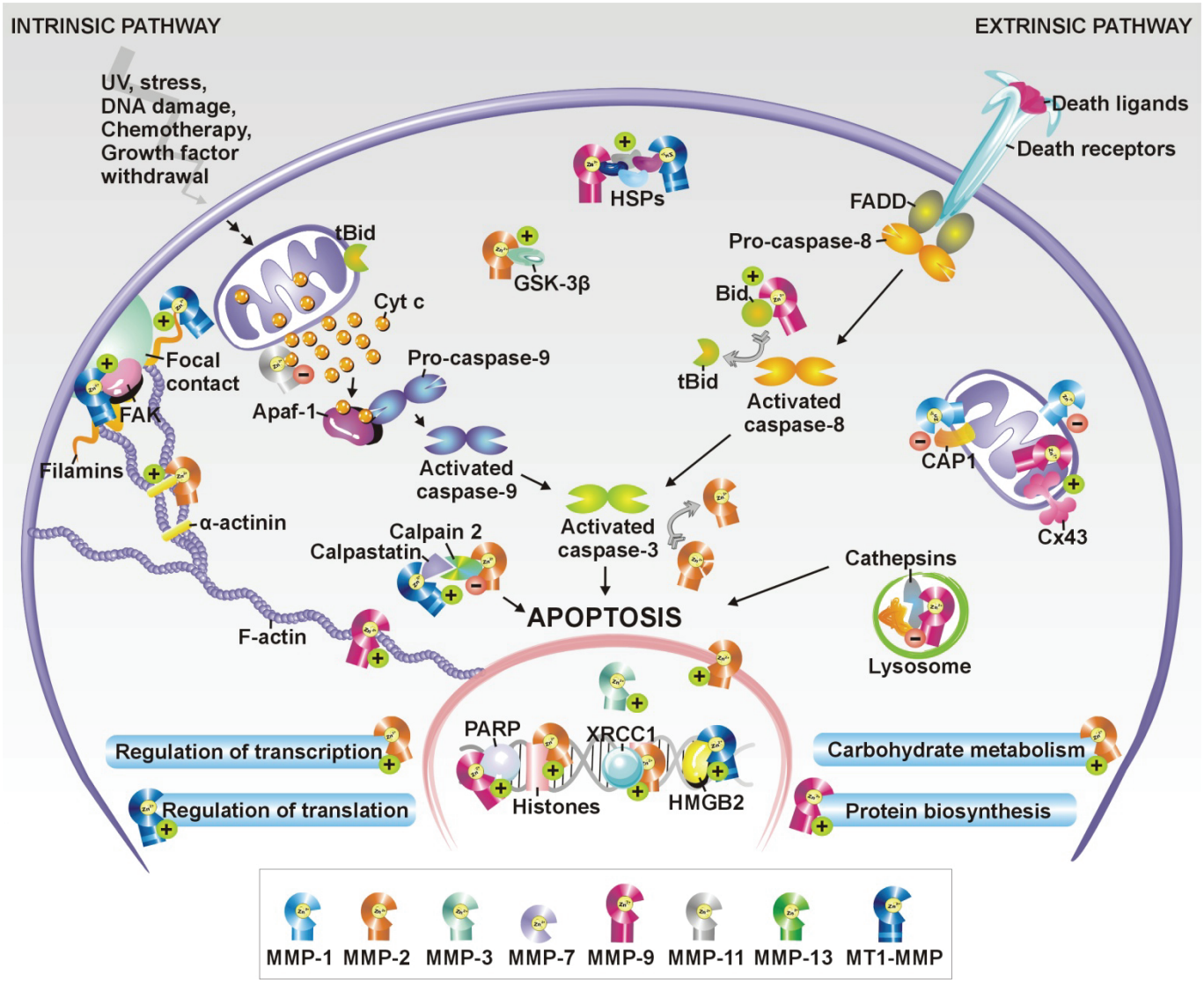

Figure 7. Intracellular proteolysis by MMPs in apoptosis. Cleavage of intracellular substrates by MMPs has many pro-apoptotic effects (+), but may also inhibit apoptotic pathways (-). Degradation of cytoskeletal proteins (e.g. focal adhesion kinase (FAK), α-actinin, actin and the filamins) contributes to the systematic dismantling of the dying cell, as well as to the disruption of the actin meshwork surrounding the nuclear lamina. Inactivation of housekeeping proteins such as the enzymes that regulate transcription and translation, carbohydrate metabolism and protein biosynthesis, may shut off life-support systems of the cell. Cleavage of heat shock proteins might abolish their multiple anti-apopototic functions. Truncation of the pro-apoptotic protein BH3-interacting death domain antagonist (Bid) may contribute to the intrinsic apoptotic pathway, in contrast with cytochrome c degradation. Translocation and degradation of connexin-43 (Cx43) in the mitochondria leads to mitophagy and cell death. Cleavage of glycogen synthase kinase-3β (GSK-3β) enhances its pro-apoptotic capacities. Inactivation of poly (ADP-ribose) polymerase (PARP) and X-ray cross-complementary factor 1 (XRCC1) causes apoptosis early after ischemia. Cleavage of histones may open up the chromatin to DNases and proteolysis of high mobility group B2 (HMGB2) liberates DNase activity. Degradation of the calpain inhibitor calpastatin may stimulate apoptosis, whereas inactivation of the pro-apoptotic proteases calpain 2 and various cathepsins may have anti-apoptotic effects. Translocation of CAP1 to the mitochondria is pro-apoptotic and its degradation may result in defective apoptosis. The presence of activated MMP-3 in the nucleus is pro-apoptotic, whereas mitochondrial MMP-1 confers resistance to apoptosis. Apaf-1, apoptotic protease activating factor-1; FADD, Fas-associated death domain-containing protein; tBid, truncated Bid.

Calpains are believed to be activated by an initial insult *via* a rise of intracellular Ca^{2+} from the ER, the mitochondria or an influx of extracellular Ca^{2+}, as caused for example by oxidative stress (Harwood *et al.*, 2005). Since MMPs are also Ca^{2+}-dependent enzymes (Tallant *et al.*, 2010) and may be activated by oxidative stress (*cf.* 1.2), they are indeed very likely to perform claimed calpain functions. Likewise, because caspase activity requires a free sulfhydryl group in the catalytic site, many caspase inhibitors target this part of the enzyme. MMPs possess an essential cysteine with a sulfhydryl group in the latent proform that is also a key regulator of enzyme activity. This suggests that caspase inhibitors may activate MMPs by interacting with the latency-confering Cys in the propeptide (Van Wart and Birkedal-Hansen, 1990).

In addition to the cleavage of common substrates in the apoptotic pathway, granzyme B directly activates

pro-caspases, whereas granzyme A induces caspase-independent cell death (Vandenabeele *et al.*, 2005). The parallellisms between MMPs and granzymes are striking, as they both are extracellular proteases that can be transported into the cell, for example by endocytic mechanisms (*cf.* 3.2.1). Conversely, whereas granzymes are mostly known for their intracellular substrates and their extracellular functions only start to gain appreciation (Boivin *et al.*, 2009), the reverse is true for MMPs. Calpains may activate or inactivate pro-caspases, whereas caspase 3 cleaves the general calpain inhibitor calpastatin. Thus caspase, calpain and granzyme cascades are tightly interrelated (Demon *et al.*, 2009; Harwood *et al.*, 2005). Of interest, calpain 2 and calpastatin were identified as MMP candidate substrates, as well as various cathepsins (*cf.* Table 3), which translocate from the lysosomal lumen in response to apoptotic stimuli and trigger apoptosis (Turk and Turk, 2009).

Besides the apparent overlap in (pro-)apoptotic substrate cleavage (*cf.* Figure 7), and inhibitor profiles, other elements hint for a participation of MMPs in the apoptotic cascade and the cross-talk with (pro-)apoptotic proteases. Nitrosative stress, which promotes apoptosis by activation of mitochondrial apoptotic pathways, inhibits caspases by S-nitrosylation (Kim *et al.*, 2002), whereas MMPs were shown to be activated by S-nitrosylation and consequently induced apoptosis (*cf.* 1.2) (Gu *et al.*, 2002). Hence, under specific conditions, such as oxidative and nitrosative stress, MMPs may indeed exert some caspase functions. In support of this, early activation of MMPs in response to oxidative stress and cleavage of the apoptotic caspase-3 substrate PARP-1 preceded caspase-3 activation in various studies (Yang *et al.*, 2010; Ruta *et al.*, 2009). As already mentioned, in stressed dopaminergic neurons, intracellular activated MMP-3 was shown to be a pro-apoptotic signaling molecule upstream of caspase-3 (but not by direct cleavage of MMP-3) (Choi *et al.*, 2008), whereas MMP-2 was found to be an apoptotic effector molecule downstream from caspase-3 in spontaneous apoptosis of endothelial cells (Shapiro *et al.*, 2010; Yarbrough *et al.*, 2010). Conversely, mitochondrial MMP-1 was shown to confer resistance to apoptosis (Limb *et al.*, 2005).

Additional data enhance the links between MMPs and apoptotic substrate conversions. Like calpastatins inhibit calpains, the tissue inhibitors of metalloproteinase (TIMPs) are natural MMP inhibitors (Brew and Nagase, 2010). TIMPs have been shown to inhibit (TIMP-1 and TIMP-2) or stimulate (TIMP-2 and TIMP-3) apoptosis and were also detected intracellularly (*cf.* Table 4). These findings suggest that the intracellular functions of TIMPs may contribute to the regulation of apoptosis by inhibiting MMP function or by other independent mechanisms. As a consequence, the extracellular effects of MMPs, for instance the cleavage of cytokines (e.g. pro-TNF-α) and cytokine receptors (Cauwe *et al.*, 2007), may work synergistically with intracellular substrate cleavages in the regulation of apoptosis.

Common (pro-)apoptotic substrates and inhibitor profiles, MMP activation and action in early apoptotic phases, and cross-talk with pro-apoptotic proteases all point to MMPs as executioners of a novel form of caspase-independent cell death. Caspases traditionally held the predominant role as prime mediators of apoptotic execution. However, recent evidence has accumulated that non-caspases, including calpains, cathepsins, granzymes and the proteasome have roles in mediating and promoting cell death (Vandenabeele *et al.*, 2005). Since modulation of apoptosis seems to be a *leitmotiv* in the intracellular functions of MMPs, they may soon be considered as the next addition to the growing list of apoptotic proteases. Further research into the influences of intracellular MMPs on both caspase-dependent and caspase-independent apoptosis may unveil new modes to prevent pathological cell death, or conversely, to induce apoptosis in cancer cells and pathological autoreactive cells.

4 INTRACELLULAR MMP SUBSTRATES DEGRADED OUTSIDE CELLS

It is increasingly recognized that a subset of proteins owns both intracellular and extracellular functions (Butler and Overall, 2009a). This phenomenon is denominated 'protein multifunctionality' and a classical example is the identity of the intracellular phosphomannose receptor with the extracellular receptor of insulin-like growth factor II (Morgan *et al.*, 1987). These functions may be very diverse and even affect physiopathology in opposite ways. For example, the upregulation of intracellular heat shock protein 70 (HSP70) is mostly cytoprotective and induces anti-apoptotic mechanisms in cells, whereas increased levels of extracellular HSP70 are generally immunostimulatory and augment the synthesis of co-stimulatory molecules and pro-inflammatory cytokine and chemokines (Asea, 2007). These multifunctional proteins may travel between intracellular and extracellular compartments in non-conventional ways. Alternatively, the intracellular protein pool may leak from the cell after necrotic loss of membrane integrity. Here, we discuss the specific circumstances in which intracellular proteins may be accessed and modulated by extracellular MMPs. Indeed, many of the intracellular (candidate) substrates in Table 3 and Table 5 have also been found extracellularly. Hence, quite opposite to the previous chapter, in which it is described how MMPs enter cells and specific subcellular compartments to cleave intracellular proteins, this chapter deals with intracellular proteins that exit the cell and are proteolysed by MMPs in the extracellular milieu.

4.1 Extracellular localization mechanisms of intracellular MMP substrates

4.1.1 Non-classical secretion of intracellular MMP substrates

The classical secretion of soluble proteins requires transportation into and through the endoplasmic reticulum (ER) and the Golgi apparatus. Signal peptides target proteins for translocation into the ER lumen. However, most intracellular proteins do not possess a signal sequence for secretion but leave the cell by ER-Golgi independent secretion pathways. Although the details of the unconventional secretory mechanisms remain elusive for most of these proteins, four main pathways have emerged as potential means of non-classical cell exit, i.e. direct membrane translocation, secretory lysosomes, multivesicular body (MVB)-derived exosomes and membrane vesicle secretion (Nickel and Seedorf, 2008; Nickel and Rabouille, 2009; Prudovsky *et al.*, 2008). Examples of intracellular MMP (candidate) substrates secreted by one of these four mechanisms are given in Table 7.

A first mechanism consists of *direct translocation across the plasma membrane*, for example by direct interaction of membrane lipids and spontaneous penetration of the lipid bilayer, as proposed for galectin-3 (Lukyanov *et al.*, 2005). Alternatively, translocation may require the cooperation of a molecular apparatus of integral and peripheral membrane proteins (e.g. galectin-1) (Schafer *et al.*, 2004; Delacour *et al.*, 2009). The *endolysosomal pathway* is a second pathway for unconventional secretion and involves the sequestration of soluble, cytoplasmic proteins into endolysosomes. These secretory lysosomes have characteristics of both lysosomes and secretory granules. The mechanisms by which cytoplasmic proteins may enter the lumen of the secretory lysosomes have not been fully elucidated yet, but may involve the highly conserved ABC cassette transport proteins, which can pump large and small molecules across membranes. A second trigger, extracellular ATP, is proposed to promote the fusion of secretory lysosomes with the cell membrane, which releases their content into the extracellular milieu (Nickel and Rabouille, 2009; Mambula *et al.*, 2007). Of interest, pro- and activated MMP-9 forms were identified in secretory lysosomes of reactive astrocytes (*cf.* 3.2.2) (Sbai *et al.*, 2010), suggesting that proteolysis may already occur before or during secretion.

A third pathway is the *secretion by exosomes derived from multivesicular bodies (MVBs)*. Exosomes are 40-100 nm vesicles, which originate from the inward budding of MVBs to form intraluminal vesicles with concomitant engulfment of cytosolic components. Although MVBs primarily deliver cytoplasmic proteins for degradation by fusion with lysosomes, they can also release internal vesicles or exosomes into the extracellular space following their fusion with the plasma membrane (Simpson *et al.*, 2008; Thery *et al.*, 2009). As can be deduced from Table 7, many MMP (candidate) substrates have been found in exosomes. Of interest, both pro- and functionally active MT1-MMP forms are present in exosomes and are ideally situated to cleave intracellular proteins upon secretion (Hakulinen *et al.*, 2008). Finally, a fourth mechanism for non-classical release of proteins is the *secretion of membrane vesicles*. In contrast with the endosomal origin of exosomes, these 'shedding vesicles' are generated by direct budding from the plasma membrane. According to their characteristics (size, density, appearance, sedimentation, lipid composition, protein markers) and cell type of origin, they have been termed ectosomes, shedding vesicles, shedding bodies, microparticles, membrane particles, exovesicles and microvesicles, without much consensus in terminology throughout the literature (Cocucci *et al.*, 2009; Nickel and Rabouille, 2009). Here, we will not differentiate between various subtypes and use the general term 'membrane vesicles'. Upon release, both exosomes and membrane vesicles circulate in the extracellular space adjacent to the site of discharge, where they can rupture and release the enclosed proteins. However, some of these vesicles travel considerable distances by diffusion within tissues and appear in biological fluids such as blood and even urine. In addition, recent data have emerged showing that they can fuse with other cells as a means of direct intercellular communication, which may play important roles in infections and immune functions, coagulation and tumor progression (Thery *et al.*, 2009; Cocucci *et al.*, 2009).

4.1.2 Exposure of intracellular substrates to extracellular MMPs by various forms of cell death

Apoptosis is characterized by a cascade of structural remodeling steps, which contribute to the progression towards cell death, but also prepare the cell for removal by phagocytes, preventing unwanted immune responses. Apoptotic cells initially become rounded and retract from neighboring cells, which is accompanied by a period of dynamic plasma membrane blebbing and culminates into the shedding of apoptotic bodies and small apoptotic blebs (Taylor *et al.*, 2008). Of interest, intracellular proteins undergo a striking redistribution during apoptosis and become concentrated in and on the surface of apoptotic blebs. Small blebs contain molecules associated with the ER and the membrane skeleton, whereas apopotic bodies are enriched with nuclear proteins (Rosen and Casciola-Rosen, 1999; Casciola-Rosen *et al.*, 1994). Whereas the shedding of apopotic bodies and blebs seems to be a means of safely breaking the cell apart into more manageable pieces for phagocytosis, the meaning of the intracellular protein translocation onto the cell surface is less evident. However, since both

calreticulin and annexin I trigger ligation and engulfment by phagocytes (Arur *et al.*, 2003; Gardai *et al.*, 2005), the surface localization of these intracellular proteins may constitute 'eat-me signals' for phagocytosis. In this way, various MMP (candidate) substrates are exposed for degradation by extracellular MMPs (*cf.* Table 7) (Schiller *et al.*, 2008; Casciola-Rosen *et al.*, 1994; Rosen and Casciola-Rosen, 1999) and will be discussed in the connected pathological context in section 4.2.

In the absence of phagocytes, or when necrotic cells outnumber the phagocytes, apoptotic cells are not cleared in time and progress to secondary necrosis. Necrotic cell death results in the discharge of the intracellular content into the extracellular milieu, and will allow for the full intracellular protein pool to come in contact with extracellular MMPs. In principle, this may be caused by any condition that destroys the cellular integrity, by physical stresses, such as heat, cold, positive or negative pressure and irradiation at various wavelengths. Chemical insults such as pH and toxins, biochemical activation of various classes of enzymes (e.g. lipases), porines (e.g. complement) and membrane channels, and finally infections with cytopathogenic viruses and other micro-organisms, all contribute to the live scenario of MMPs acting on intracellular substrates. Furthermore, some intracellular proteins are released from necrotic cells before the loss of membrane integrity. These specific damage-associated molecular patterns (DAMPs) or 'alarmins' are released from primary and secondary necrotic cells, but not from apoptotic cells. Hence, they function as endogenous adjuvants and activate the innate and adaptive immune system, signaling the 'danger' of immunogenic cell death and tissue damage (Kono and Rock, 2008; Bianchi, 2007). Various proteins have been proposed to function as alarmins, many of which were identified as MMP (candidate) substrates (*cf.* Table 7) (Kono and Rock, 2008; Yang *et al.*, 2009; Bianchi, 2007). Modulation of these pro-inflammatory molecules may account for some of the multiple immunomodulatory roles of MMPs (Cauwe *et al.*, 2007; Manicone and McGuire, 2008). Indeed, hyperactivation by MMP-mediated cleavage would strongly enhance inflammation, whereas an inactivating proteolytic effect may be required for the termination of the pro-inflammatory effect, much alike the thrombin-mediated cleavage and dampening of HMGB1 activity (Ito *et al.*, 2008).

Although apoptosis and necrosis seemed to be the two fates of choice for a dying cell, it has recently become clear that some phagocytic cells also succumb via the formation of extracellular chromatin structures, which were first identified in neutrophils and were termed neutrophil extracellular traps or NETs (Brinkmann *et al.*, 2004). Upon activation by pro-inflammatory stimuli (IL-8, lipopolysaccharide (LPS), bacteria, fungi, activated platelets), neutrophils start a program that leads to the formation of NETs and to their death, termed NETosis. This involves gradual disintegration of the nuclear membranes and loss of granule integrity, with subsequent filling of the cell with nuclear material, mixed with cytoplasmic and granule contents. Hence, these NETs consist of bundled chromatin fibers (DNA and histones), decorated with (antimicrobial) granular and cytoplasmic proteins. NETs capture and kill microbes such as bacteria, fungi and parasites using these antimicrobial proteins as well as bactericidal histones (Brinkmann and Zychlinsky, 2007; Papayannopoulos and Zychlinsky, 2009). Indeed, histones and histone-derived fragments were shown to have a variety of antimicrobial functions, including bacterial cell membrane permeabilization, penetration into the membrane, and binding and neutralizion of bacterial LPS toxicity. Of interest, a portion of these histone fragments have been shown to be produced from precursor histones *via* specific cleavage by endogenous proteases (Kawasaki and Iwamuro, 2008). Since histones were identified as MMP-2 candidate substrates (*cf.* Table 3), and endogenous or exogenous ROS are required for NET formation and may activate MMPs (*cf.* section 1.2), MMPs may very well be the activators of histone microbicidal activity, intracellularly before the NETs are released, or extracellularly after NET expulsion. This would indeed be very similar to the respective intracellular and extracellular activation of murine and human α-defensins, as discussed in section 3.3.1. MMP-9, which is present in tertiary neutrophil granules, was also found on these NETs (Brinkmann *et al.*, 2004), and is thus ideally positioned to participate in proteolytic defensin and histone activation. A recent proteomic study identified 24 different NET-associated proteins, a surprisingly restricted set, since the chromatin comes in contact with the full cytoplasmic contents during NETosis and the cell membrane ruptures during NET release (Urban *et al.*, 2009). Interestingly, half of these NET proteins are MMP (candidate) substrates (*cf.* Table 7), again pointing to the potential modification of NET activities by MMPs. NETosis is restricted to neutrophils, but the formation of different kinds of extracellular traps (ETs) was also observed in other granulocyte cell types, such as mast cells and eosinophils (von Kockritz-Blickwede and Nizet, 2009).

In conclusion, whereas MMPs may cleave various substrates within cells, it is clear that they can also access many intracellular proteins in the extracellular milieu. Since many of the proteins that leave cells by unconventional ways are molecules with multiple functions, that differ according to their location in the extracellular *vs*. intracellular milieu, the modulation of these substrates by MMPs adds an additional layer of complexity to MMP inhibition in pathology. Indeed,

inhibition of potential detrimental effects of MMPs on these bimodal substrates will require milieu-specific inhibitors that do not enter the compartment where substrate cleavage is beneficial.

Table 7. Extracellular localization mechanisms of intracellular MMP substrates

NON-CLASSICAL SECRETION MECHANISMS	
DIRECT TRANSMEMBRANE TRANSLOCATION	
Galectin-1	(Schafer *et al.*, 2004)
Galectin-3	(Lukyanov *et al.*, 2005)
LYSOSOMAL SECRETION	
Cathepsin D	(Mambula *et al.*, 2007)
HMGB1	(Gardella *et al.*, 2002)
HSP70	(Mambula *et al.*, 2007)
EXOSOME-MEDIATED SECRETION	
Actin-β/γ	(Thery *et al.*, 2001)
α-Actinin-4	(Simpson *et al.*, 2008)
Alix	(Thery *et al.*, 2001)
Annexin I	(Thery *et al.*, 2001)
Carbonic anhydrase II	(Simpson *et al.*, 2008)
Cofilin-1	(Thery *et al.*, 2001)
Cyclophilin A	(Yu *et al.*, 2006)
EF 1-α1	(Yu *et al.*, 2006; Thery *et al.*, 2001)
EF2	(Thery *et al.*, 2009)
Enolase-α	(Yu *et al.*, 2006)
Ezrin	(Thery *et al.*, 2009; Simpson *et al.*, 2008)
Ferritin light chain	(Thery *et al.*, 2001)
GAPDH	(Thery *et al.*, 2009; Simpson *et al.*, 2008)
Galectin-3	(Thery *et al.*, 2001)
Histone H2A	(Thery *et al.*, 2001)
Histone H2B	(Thery *et al.*, 2001)
Histone H4	(Thery *et al.*, 2001)
HSC70	(Thery *et al.*, 2009; Simpson *et al.*, 2008)
HSP70	(Thery *et al.*, 2009)
HSP90	(Thery *et al.*, 2001; Yu *et al.*, 2006)
Malate dehydrogenase	(Simpson *et al.*, 2008)
Moesin	(Thery *et al.*, 2009; Simpson *et al.*, 2008)
Myosin	(Thery *et al.*, 2009; Simpson *et al.*, 2008)
Peroxiredoxin-1	(Thery *et al.*, 2001)
PGK1	(Yu *et al.*, 2006)
PGM1	(Simpson *et al.*, 2008)
Profilin-1	(Thery *et al.*, 2001)
Rab GDI-β	(Simpson *et al.*, 2008)
Rho GDI-α/β	(Thery *et al.*, 2009; Simpson *et al.*, 2008)
Tubulin-α/β	(Thery *et al.*, 2001)
14-3-3 proteins	(Thery *et al.*, 2001; Simpson *et al.*, 2008)
SECRETION BY VESICLE SHEDDING	
Annexin I	(Simpson *et al.*, 2008)
Galectin-1	(Cooper and Barondes, 1990; Harrison and Wilson, 1992)
Galectin-3	(Simpson *et al.*, 2008; Mehul and Hughes, 1997)
Histones	(Simpson *et al.*, 2008)
HSP27	(Simpson *et al.*, 2008)
HSP70	(Simpson *et al.*, 2008)
HSP90	(Simpson *et al.*, 2008)

CELLULAR EXIT DURING CELL DEATH	
APOPTOTIC BLEBS/BODIES	
Annexin I	(Arur *et al.*, 2003)
La	(Casciola-Rosen *et al.*, 1994)
Calreticulin	(Rosen and Casciola-Rosen, 1999)
Histone H2A	(Schiller *et al.*, 2008)
Histone H2B	(Schiller *et al.*, 2008)
Histone H4	(Schiller *et al.*, 2008)
Jo-1	(Rosen and Casciola-Rosen, 1999)
PARP	(Rosen and Casciola-Rosen, 1999)
Vimentin	(Boilard *et al.*, 2003)
ALARMINS	
α-defensins	(Yang *et al.*, 2009)
Annexin I	(Bianchi, 2007)
Galectin-1	(Cambi and Figdor, 2009)
Galectin-3	(Cambi and Figdor, 2009)
HDGF	(Bianchi, 2007)
HMGB1	(Bianchi and Manfredi, 2007)
HSP70	(Bianchi, 2007; Kono and Rock, 2008)
HSP90	(Bianchi, 2007; Kono and Rock, 2008)
Nucleolin	(Bianchi, 2007)
NETOSIS	
Actin-β/γ	(Brinkmann *et al.*, 2004; Urban *et al.*, 2009)
α-Actinin-1	(Urban *et al.*, 2009)
α-Actinin-4	(Urban *et al.*, 2009)
α-defensins	(Urban *et al.*, 2009)
Annexin I	(Brinkmann *et al.*, 2004)
Cytochrome c	(Brinkmann *et al.*, 2004)
Enolase-α	(Urban *et al.*, 2009)
Histone H2A	(Brinkmann *et al.*, 2004)
Histone H2B	(Brinkmann *et al.*, 2004)
Histone H4	(Brinkmann *et al.*, 2004)
Myosin	(Urban *et al.*, 2009)
Tubulin-α	(Brinkmann *et al.*, 2004)

The meaning of the acronyms can be found in Table 3 and 5.

4.2 Extracellular proteolysis of intracellular substrates in physiology and pathology

4.2.1 Extracellular proteolysis of intracellular autoantigens in organ-specific autoimmune diseases: multiple sclerosis

Multiple sclerosis (MS) is a chronic neurological disorder of the CNS, characterized by the breakdown of the BBB, perivascular infiltration of inflammatory cells, leading to the formation of the so-called 'vascular cuffs', and multiple regions of focal myelin and neuronal loss (lesions or plaques). MMPs may contribute to these pathogenic events by increasing the permeability of the BBB, enhancing demyelination by the degradation of myelin (glyco) proteins, e.g. myelin basic protein (MBP). By the generation of antigenic peptides, and by the facilitation of infiltration and migration of immune cells through the ECM and the basal membrane (Cuzner and Opdenakker, 1999; Opdenakker and Van Damme, 1994; Rosenberg, 2009; Agrawal *et al.*, 2006; Opdenakker *et al.*, 2003). While attempting to identify the components of the myelin sheath that provoke the autoimmune reaction in MS, van Noort and colleagues isolated αB-crystallin (αB-crys) as a prominent target in myelin from MS brains, but not in healthy brain myelin (van Noort *et al.*, 1995). In addition, αB-crys is the most abundant gene transcript present in early active MS lesions, whereas such transcripts are absent in normal brain tissue (Chabas *et al.*, 2001). MMP-9 cleaves αB-crys at multiple sites *in vitro* (*cf.* Table 8) and released various immunodominant and cryptic epitopes of αB-crys. Whereas both intact αB-crys and the immunodominant peptide 1-16 stimulated T-cell proliferation *in vitro*, neither intracerebral injection of the MMP-9-generated fragments nor injection of intact αB-crys triggered immediate neuroinflammation in an unprimed host *in vivo* (Starckx *et al.*, 2003). Besides being a major MS autoantigen, αB-crys was discovered to possess neuroprotective and anti-apoptotic functions in the brain. αB-crys knockout mice are more susceptible to development of experimental autoimmune encephalomyelitis (EAE), a murine MS model, and administration of recombinant αB-crys ameliorated EAE symptoms (Ousman *et al.*, 2007). Hence, degradation of αB-crys by MMP-9 may be pathogenic by the ablation of these protective functions (Starckx *et al.*, 2003). This is supported by the finding that young gelatinase B-deficient mice are resistant to EAE development (Dubois *et al.*, 1999). The loss of protection at a more advanced age may be explained by the fact that various MMPs proteolyse αB-crys at multiple scissile bonds (*cf.* Table 8) (Shiryaev *et al.*, 2009) and may compensate for the absence of MMP-9-mediated αB-crys destruction.

Table 8. Overview of the cleavage sites of various MMPs in αB-crystallin

Cleavage sites*	MMP-9†	MMP-2	MMP-8	MMP-10	MMP-12	MT1-MMP	MT2-MMP	MT3-MMP	MT4-MMP	MT5-MMP	MT6-MMP
His7-Pro8	√						√	√			
Pro16-Phe17	√										
Arg22-Leu23	√	√									√
Pro46-Phe47	√	√	√	√	√	√	√	√		√	√
Tyr48-Leu49	√		√		√	√				√	
Ser53-Phe54				√		√	√	√			√
Phe54-Leu55	√	√	√	√	√	√	√	√	√	√	√
Trp60-Phe61	√	√		√		√	√	√			√
Glu67-Met68	√	√				√	√	√	√		√
His83-Phe84											√
Glu88-Leu89	√	√	√					√	√		√
Asp96-Val97											√
Phe113-Ile114		√		√	√	√	√	√			√
Lys121-Tyr122	√	√	√		√			√			√
Pro130-Leu131	√						√	√			√
Tyr132-Ile133	√	√	√	√	√	√	√	√	√		√
Lys150-Gln151	√	√	√	√			√	√	√	√	√

*The cleavage sites are compiled from (Shiryaev *et al.*, 2009) and (Starckx *et al.*, 2003).

†MMP-9 also partially processes the following peptide bonds: Met1-Asp2; Asp2-Ile3; Ala4-Ile5; Asp25-Gln26; Glu30-His31; Leu32-Leu33; Ser35-Asp36; Thr40-Ser41; Thr42-Ser43; Phe47-Tyr48; Arg69-Leu70; Asn78-Leu79; Glu99-Val100; Val100-His101; Gly102-Lys103; Gly112-Phe113; Tyr122- Arg123; Ser136-Leu137; Pro160-Ile161;Val169-Tyr170 (Starckx *et al.*, 2003).

4.2.2 Extracellular proteolysis of intracellular autoantigens in systemic autoimmune diseases: SLE

Systemic lupus erythematosus (SLE) is a prototypic systemic autoimmune disease characterized by the production of high titers of autoantibodies directed against ubiquitous intracellular, and mostly nuclear, autoantigens. The diverse symptoms of SLE range from rash and arthritis through anemia and thrombocytopenia to serositis, nephritis, seizures, and psychosis. Pathogenic autoantibodies and circulating immune complexes are the primary cause of tissue damage in patients with SLE (Rahman and Isenberg, 2008). More than 100 different autoantibodies have been found in SLE patients, and these arise from the dysregulation of all of the key components of the immune system (Sherer *et al.*, 2004). Apoptotic cells have been increasingly accepted to have initiating and propagating effects in SLE (Munoz *et al.*, 2005). Indeed, lymphocytes of SLE patients show accelereated apoptotis rates (Emlen *et al.*, 1994; Denny *et al.*, 2006). In addition, in SLE, phagocytes have decreased phagocytic activities, which results in a defective clearance of apoptotic cells (Ren *et al.*, 2003; Gaipl *et al.*, 2005). Hence, the overload of apoptotic cells progresses to secondary necrosis and intracellular proteins become accessible to post-translational modifications (Dieker and Muller, 2009), for example by proteases (*cf.* 4.1.2). Altered intracellular autoantigens and danger signals released from necrotic cells create a pro-inflammatory environment in which dendritic cells engulfing apoptotic cells may become activated instead of tolerogenic (Savill *et al.*, 2002; Viorritto *et al.*, 2007). These activated 'immunogenic' dendritic cells may in turn activate autoreactive T and B cells, leading to the production of autoantibodies and formation of pathogenic immune complexes. Infection or environmental exposures may raise the apoptotic burden to initiate autoimmune disease and cause relapses. Furthermore, apoptotic cells cluster intracellular and nuclear proteins on the surface of apoptotic blebs and apoptotic bodies (*cf.* 4.1.2), many of which are major SLE autoantigens.

Hence, the question was raised whether MMPs may modify these systemic autoantigens exposed on the apoptotic cell surface, as well as the overload of SLE autoantigens released during (secondary) necrosis. To investigate this, a system of dying monocytic leukemia cells was used with MMP-9 as a model protease. This 'one-dimensional degradomics system' identified adenylyl cyclase-associated protein-1 or CAP1 as novel and highly efficient substrate of MMP-9 during cellular necrosis (Cauwe *et al.*, 2008). Indeed, cleavage of CAP1 was more efficient than the cleavage of gelatin, the physiological MMP-9 substrate. In addition, CAP1 was also degraded by other MMPs (*cf.* Table 5), but at physiological concentrations, MMP-9 was the only (tested) MMP that caused its degradation. CAP1 is a cytoskeletal protein involved in the promotion of actin filament turnover (Bertling *et al.*, 2004; Moriyama and Yahara, 2002) and it was identified as an autoantigen in SLE (Frampton *et al.*, 2000) and rheumatoid arthritis (Kinloch *et al.*, 2005). Moreover, intact CAP1 was identified in the urines of patients with systemic autoimmune diseases such as SLE, vasculitis and Sjögren's syndrome, as well as in healthy control urines. Whereas healthy control urines did not contain activated MMP-9, urine samples of patients with clinical parameters suggesting renal failure showed increased levels of pro-MMP-2 and pro-MMP-9, and the appearance of activated forms of both gelatinases, as evidenced by substrate zymography. Of interest, in some patient urines, an inverse relation was observed between the levels of intact CAP1 and activated forms of MMP-2 and MMP-9, and one urine sample of an SLE patient contained CAP1 fragments. These findings, together with the high turnover rate of CAP1 by MMP-9, suggest that this cleavage may occur *in vivo* (Cauwe *et al.*, 2008). In a recent study, a pro-apoptotic role is described for CAP1. Upon apoptosis induction by various stimuli, CAP1 translocates rapidly to the outer mitochondrial membrane even before caspase activation. CAP1-knockdown cells are resistant to apoptosis inducers, showing that the mitochondrial translocation of CAP1 is proapoptotic, possibly by shuttling apoptosis-inducing actin to the mitochondria (Wang *et al.*, 2008). MMP-9 was found in mitochondria (*cf.* 3.2.5 and 3.3.3) and its efficient cleavage of CAP1 may result in defective apoptosis, pushing the cell death program towards immunogenic necrosis. Alternatively, CAP1 may be cleaved by MMP-1, which was found to confer resistance to apoptosis when associated with mitochondrial membranes (*cf.* sections 3.2.5 and 3.3.6 and Figure 7) (Limb *et al.*, 2005).

By developing and applying a two-dimensional degradomics (2DD) approach to THP-1 cytosol (*cf.* chapter 2), the intracellular degradome of MMP-9 was further expanded and this demonstrated that about two thirds of the identified (candidate) substrates are autoantigens described in one or multiple autoimmune conditions, and in cancer (e.g. annexin I, nucleolin, citrate synthase, cyclophilin A, HMGB1/2, α-enolase, histidyl-tRNA synthetase, HSP27, HSP90, phosphoglycerate kinase 1) (Cauwe *et al.*, 2009). From Tables 3 and 5, it is clear that cleavage of systemic autoantigens may be a general MMP function, and that cleavage by MMPs is predictive of autoantigen status, as was described for the caspases and granzyme B (Rosen and Casciola-Rosen, 1999). Indeed, on top of the above-mentioned autoantigens targeted by MMP-9, MMPs (may) cleave many nucleic acid-associated autoantigens such as histones, hnRNPs, La, PARP-1, SmD3, nucleophosmin and cytoplasmic autoantigens including various elongation factors, cytoskeletal proteins, calpastatin, cathepsins G (Sherer *et al.*, 2004; Hoffmann *et al.*, 2009; Cauwe *et al.*, 2009).

Proteolysis of systemic autoantigens by caspases and granzymes was proposed to be immunogenic by the release of normally 'hidden' cryptic epitopes (Rosen and Casciola-Rosen, 1999). However, Granzyme B may both release and destroy immunodominant epitopes (Darrah and Rosen, 2010). In addition, a study with granzyme B-deficient mice showed that granzyme B is not required for the development of pristane-induced SLE and may even have a protective effect, as the granzyme B-deficient mice showed increased mortality after pristane treatment (Graham *et al.*, 2005). This suggests that autoantigen cleavage does not necessarily lead to the stimulation of autoimmunity. As cleavage by MMPs is also predictive for autoantigen status, a similar question remains. Will proteolysis by MMPs lead to the release of tolerance-breaking neo-epitopes or will degradation by MMPs disrupt immunodominant epitopes and contribute to the silent removal of (abundant) intracellular proteins after necrosis and tissue injury?

In addition, the complex consequences of systemic autoantigen cleavage by MMPs may not be restricted to the alteration of the substrate's immunogenicity. Indeed, by cleavage of calreticulin and annexin I at the surface of apopotic cells, MMPs may abolish recognition by phagocytes in SLE (*cf.* 4.1.2), contribute to the clearance deficiency and further tip the balance to immunogenic secondary necrosis. Alternatively, ingestion of apoptotic cells has been suggested to be tolerogenic in the absence, and immunogenic in the presence of danger signals, respectively (Viorritto *et al.*, 2007; Savill *et al.*, 2002). Hence, if MMPs degrade and inactivate danger signals, as discussed for the above-mentioned alarmins (*cf.* 4.1.2), they may promote tolerance and dampening of inflammation.

In conclusion, further examination of the effects of proteolysis by MMPs on the immunogenicity and function of systemic autoantigens may yield interesting insights into the etiology and relapsing-remitting mechanisms of complex systemic autoimmune diseases.

4.2.3 Extracellular proteolysis of intracellular autoantigens in acute necrotic conditions

Fulminant hepatic necrosis, septic shock, ischemic conditions, acute respiratory distress syndrome (ARDS), tumor lysis syndrome, pre-eclampsia during pregnancy, hemolysis due to malaria, severe traumatic and burn injuries, are all acute conditions accompanied by massive cellular necrosis and tissue injury. These conditions depend on a rapid 'cleaning' system to remove the overload of toxic and immunogenic proteins released from the cells, in order to prevent inflammation and secondary injury. Indeed, when cells die, monomeric and filamentous actins are released into the extracellular space and reach the systemic circulation. In the plasma, where the ionic strength, pH and temperature promote polymerization, actin monomers can form long filaments together with coagulation factor Va, which triggers disseminated intravascular coagulation, if not rapidly resolved. Functional organ decompensation leads to a condition resembling multiple organ dysfunction syndrome (MODS). In addition, the high viscosity of actin filaments, the inhibitory effect of actin on fibrinolysis, and the fact that actin binds adenine nucleotides that activate purinergic receptors are all mechanisms that may lead to secondary tissue injury (Bucki *et al.*, 2008). However, to avoid these toxic effects of extracellular actin, a complex actin-scavenging system exists in the vascular compartment. This system involves 2 proteins: plasma gelsolin that releases monomers from the toxic filaments, and Gc-globulin, which complexes the freed monomers. Both actin-gelsolin and actin-Gc-globulin complexes are subsequently cleared by the liver phagocytes much more efficiently than the free proteins (half-lives of 30 min. *vs.* 1-2 days, respectively). Nevertheless, excessive release of cellular actins or decreased activity of the actin-scavenger system causes severe pathological conditions such as MODS, hepatic necrosis, ARDS, septic shock, and complications of pregnancy (Haddad *et al.*, 1990; Lee and Galbraith, 1992; Dahl, 2005; Meier *et al.*, 2006).

Gelsolin exists in an intracellular and a secreted isoform. Both isoforms are derived from a single gene by alternative transcriptional initiation sites and mRNA processing, which removes the signal sequence and NH_2-terminus to generate cytoplasmic gelsolin. The term 'gelsolin' refers to its ability to convert filamentous actin (F-actin) from a 'gel' to a 'solvent' state by rapid shortening of the filaments (Kwiatkowski *et al.*, 1988). Plasma gelsolin not only binds actin, but also scavenges a variety of potentially inflammatory moieties such as platelet-activating factor, lysophosphatidic acid, sphingosine-1-phosphate and bacterial cell wall constituents. In this way, gelsolin can modulate the exuberance of the host response to sepsis, malaria, burns, trauma, and other acute clinical conditions. The lower the levels of plasma gelsolin, the less favorable the prognosis of acute illness becomes (DiNubile, 2008; Bucki *et al.*, 2008). Plasma gelsolin was identified by 2D-PAGE as a MT1-MMP substrate in plasma (Hwang *et al.*, 2004). In addition to MT1-MMP, gelsolin is cut into several fragments by various MMPs. MMP-3 cleaves gelsolin most efficiently, followed by MMP-2, MMP-1, MT1-MMP and MMP-9. MMP-3 cleaves gelsolin at Asn416-Val417, Ser51-Met52, and Ala435-Gln436, which results in considerable loss of its depolymerizing activity (Hwang *et al.*, 2004; Park *et al.*, 2006). This suggests that MMPs may weaken the extracellular actin-scavenging system by cleaving gelsolin and enhancing primary and secondary injury in pathological conditions induced by extracellular actin.

However, MMPs may also have a beneficial effect after massive necrosis. Indeed, by cleaving actin, they diminish primary and secondary effects of actin toxicity. By degradation of many essential

actin/tubulin-binding proteins such as ezrin, moesin, CAP1, Arp2/3 complex subunits, IQGAP1, profilin, stathmin, tubulin (*cf.* Tables 3 and 5), they may inhibit polymerization and branching of the cytoskeleton in the extracellular space and prevent the previously mentioned consequences of massive necrosis (Cauwe *et al.*, 2009). Hence, cleavage of cytoskeleton-associated proteins after necrosis may have both positive and negative outcomes. The extracellular clearance may be part of a physiological process that clears proteins released by occasional necrosis in the tissues. In steady-state conditions, MMP activity in the circulation is kept in check by the general inhibitor α_2-macroglobulin. Hence, gelsolin will not be cleaved and may exert its actin-scavenging role. However, in acute inflammation, activated MMPs are found in the circulation, where they may inactivate gelsolin with the ensuing pathological consequences. In addition, the outcome may depend on the MMP in charge. Since MMP-9 is less efficient at gelsolin cleavage, it may have mostly beneficial effects by clearing abundant cytoskeletal proteins and by the very efficient degradation of CAP1 (*vide supra*). However, MMP-3 may be mostly pathogenic by weakening the gelsolin scavenging capacity. Since MMP inhibitors were proposed as an interesting line of therapy in acute inflammatory conditions (Hu *et al.*, 2007), determining the balance of pathogenic and beneficial effects of MMPs after massive necrosis may be of critical importance. Indeed, an ideal time-window and duration of inhibition will limit or even prevent toxic side effects (Hu *et al.*, 2007).

4.2.4 Extracellular proteolysis of intracellular autoantigens in amyloid diseases

Protein conformational diseases are diverse disorders characterized by abnormal unfolding, followed by aggregation and progressive accumulation of a disease-associated (glyco)protein. Another common feature of these protein conformational diseases is that they are mostly late-onset illneses (Surguchev and Surguchov, 2010). The pro-aggregating roles of MMPs in two protein conformational diseases with mostly intracellular accumulation of protein aggregates, namely Parkinson's disease (*cf.* 3.3.4) and cataract (*cf.* 3.3.5), were already discussed. Here, we discuss the aggregation of gelsolin, which leads to familial amyloidosis of Finnish type (FAF). FAF is a late-onset autosomal dominant disease, which leads to progressive peripheral neuropathy that involves the cranial nerves and especially the facial nerve. These symptoms are caused by deposition of amyloid in the perineurium, vascular walls, cornea and skin (Luttmann *et al.*, 2010). FAF patients possess mutated plasma gelsolin (D187Y/N), which is aberrantly processed by at least two successive proteolytic events to generate amyloidogenic peptides. D187Y/N mutations abolish Ca^{2+} binding in domain 2, destabilizing and rendering the domain accessible to aberrant proteolysis by an α-gelsolinase, that was identified as furin (Chen *et al.*, 2001b). Cleavage of mutated gelsolin by furin at Arg172-Ala173 occurs as it transits through the Golgi apparatus and yields a secreted 68 kDa COOH-terminal fragment (C68) that contains the amyloidogenic region at is NH_2-terminus. C68 is the substrate for a second protease, β-gelsolinase, whose activity yields major 8 kDa and minor 5 kDa amyloidogenic fragments. Incubation of C68 with lysates of the human fibrosarcoma-derived cell lin HT1080 (but not with HT1080 culture medium) resulted in cleavage of C68 into the amyloidogenic 8 kDa and 5 kDa peptides, and this was inhibited by the general MMP inhibitor GM6001 and TIMP-2, but not by TIMP-1. These results suggest that the β-gelsolinase is an MT-MMP. Indeed MT1-MMP cleaves C68 *in vitro* at Ala242-Met243, generating the 8 kDa fragment, which is converted into the 5 kDa upon additional incubation with MT1-MMP (Page *et al.*, 2005). Other MMPs also cleave C68 *in vitro* (*cf.* 4.2.3), i.e. MMP-3, -7, -9, but not MMP-2, indicating that multiple MMPs may contribute to amyloidogenesis. ECM components, such as glycosaminoglycans accelerate amyloidogenesis at neutral pH, suggesting that gelsolin is cleaved and forms fibrils in the neutral environment of the ECM (Annabi *et al.*, 2001). However, since amyloidogenesis is optimal at low pH (Ratnaswamy *et al.*, 1999) and MMPs may be activated intracellularly, intracellular β-gelsolinase activity by MMPs can not be excluded.

MMPs were also shown to be involved in fibril formation (and degradation) in AA amyloidosis, caused by aggregation of the acute-phase protein serum amyloid A (SAA) (Stix *et al.*, 2001) and in the most prevalent amyloid disease, Alzheimer's disease, caused by amyloid β (Aβ) deposition in the brain. Of interest, gelsolin inhibits the fibril formation of Aβ and destabilizes preformed Aβ fibrils (Ray *et al.*, 2000). In addition, cytoplasmic gelsolin reduces Aβ burden in a mouse model of Alzheimer's disease and prevents Aβ-induced apoptotic mitochondrial changes (Antequera *et al.*, 2009). Hence, cleavage of the anti-amyloidogenic gelsolin may have pathogenic effects in both FAF, Alzheimer's disease, and in acute necrotic conditions (*cf.* 4.2.3).

In conclusion, the clarification of the roles played by MMPs in these and other amyloid diseases and protein conformational disorders, may result in novel therapeutic strategies.

CONCLUSION

Matrix metalloproteinase substrate identification has evolved from extracellur matrix molecules to secreted and membrane-bound molecules. All these substrates imply the extracellular action of MMPs. Here, we review that intracellular localization of MMPs may not be an artifact by mislocalizations. Activated MMPs are found in many subcellular compartments where they cleave intracellular proteins or even exhibit non-proteolytic functions acting as transcription factor (Eguchi *et al.*, 2008) or antimicrobial agent (Houghton *et al.*, 2009). Indeed, most MMPs are modular entities that often share protein domains with members of sister enzyme families, including the 'a disintegrin and metalloproteinase (ADAM)-family' and the 'a disintegrin and metalloproteinase with thrombospondin-like motif (ADAMTS)' enzymes. Therefore, it is not surprising that biological functions of specific MMPs depend on protein domains different from the catalytic site. A recent prominent example of such MMP multifunctionality is the anti-apoptotic activity of the hemopexin domain, discovered for human MMP-2 and MMP-9 (Redondo-Munoz *et al.*, 2010) and the non-catalytical unwinding of collagen by collagenases and aggrecanases (Nagase and Fushimi, 2008) .

Searching for common denominators in the intracellular actions of MMPs, we came across various interrelated pathophysiological settings, such as cancer, cellular stress, apoptosis, degenerative and protein conformational diseases. Indeed, under cellular stress, MMPs may be activated by ROS and RNS, and exert pathological actions such as the cleavage of heat shock proteins and other chaperones that are upregulated to avoid stress-induced protein precipitation and that rescue a stressed cell from apoptosis. Hence, cleavage of chaperones is pro-apoptotic and will lead to protein aggregation and cell death, as observed in protein conformational diseases, such as cataract, Parkinson's disease and amyloid disorders. Proteolysis by MMPs even causes direct aggregation and precipitation of amyloidogenic proteins, suggesting that MMP inhibition in such protein conformational diseases may have double benefits. Extended cell death leads to degeneration, for example after ischemia/reperfusion injury in heart and brain. Many observed intracellular cleavages have turned out to have pro-apoptotic effects. However, dual roles in the apoptotic cascade are to be expected. Understanding the roles that MMPs may play in the decision making process as to whether a cell should live or die, may shed new light on the targeting of cancer cells and autoreactive cells in autoimmunity, and the prevention of cell death in degenerative diseases. As a result, an additional reason why MMPs are produced as inactive zymogens seems to be to avoid intracellular damage in healthy cells, whereas stressed cells have various ways to activate the MMPs, such as proteolysis by caspases and oxidative stress. However, as MMP activity is regulated by subtle concentration changes of ROS and RNS, as well as by phosphorylation, it cannot be excluded that MMPs also function as intracellular signaling molecules in cellular homeostasis, by proteolytic or non-proteolytic actions.

It will presumably take a while before we understand the complexities of subcellular enzyme substrate ecosystems in e.g. inflammation, at the tumor cell invasion front or in a developing organism. Along similar lines of complexities, by degradomics and reverse-degradomics approaches, attempts are made to understand the complex protease web (Overall and Kleifeld, 2006; Kruger *et al.*, 2010). Similar to the Human Genome (HUGO) Project, the elucidation of degradomes, the establishment of the protease web and the exact and the complete remodeling of any protein as a substrate of proteases and peptidases are technically doable. Whether the full insights will lead to new treatments, remains an unanswered question. However, the definition of a critical activation cleavage within a cascade or a network will remain a valuable approach for the development of novel therapies.

Nevertheless, therapeutic MMP inhibition is still far from home. The well-acknowledged complexities are the extended redundancy within the MMP family for a specific proteolytic action, with another MMP compensating for the inhibited one. The extensive lists of substrates discovered for every single MMP imply that pathogenic substrates will accumulate when inhibiting cleavage of physiological ones. In addition, the same MMP may have a pathological effect in an early disease state and a protective function in the final stages. Hence, inhibitors should not only be MMP-specific, but also time- and substrate-specific. With the discovery of intracellular MMP functions, an additional dimension is added to tissue ecosystems, since MMPs inside or outside cells may have different and even opposite effects on a particular disease outcome. Hence and ideally, an effective MMP inhibitor should also be specific for the specific subcellular locations where the particular MMP action is pathogenic.

In conclusion, what we presently know about intracellular MMP functions and cleavages of intracellular substrates may only be the tip of the iceberg. Further characterization of intracellular substrates in physiology and pathology is amongst the challenges in current MMP research.

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DISCUSSION PART 2. NOVEL FUNCTIONS OF GELATINASE B/MMP-9 IN THE SUPPRESSION OF LYMPHOPROLIFERATION AND SYSTEMIC AUTOIMMUNITY

Inbred mice that spontaneously develop a disease similar to human SLE, such as New Zealand, MRL and BXSB mice, represent invaluable tools for the genetic dissection of susceptibility to SLE and for the elucidation of immunological abnormalities. Common characteristics of these mice include a generalized B cell hyperreactivity leading to hypergammaglobulinemia, early IgM to IgG switching, production of autoantibodies against a wide variety of self antigens, formation of immune complexes (ICs) and multiple histopathological manifestations, of which glomerulonephritis is the most prominent [76]. During the present doctoral research we chose to investigate the influence of MMP-9 on the homozygous *lpr* mutation in the apoptosis-inducing receptor Fas. The trimeric transmembrane receptor Fas (CD95, Apo-1) is constitutively expressed by different cell types, including T cells. In contrast, the expression of Fas ligand (FasL, CD95L, CD178) is tightly regulated and limited to immune privileged sites and activated immune cells, such as T cells after repeated T cell receptor (TCR) stimulation at the end of the immune response. Fas engagement by FasL leads to the recruitment of Fas-associated death domain-containing protein (FADD) and activation of effector caspases that cleave essential cellular proteins and DNA, leading to apoptosis [77]. On the autoimmune MRL background ($MRL^{lpr/lpr}$), the *lpr* mutation in the *Fas* gene causes IC-mediated glomerulonephritis, arthritis, vasculitis, pneumonitis and sialoadenitis, which is very similar to the autoimmune symptoms caused by the *generalized lymphoproliferative disease* (*gld*) mutation in the *FasL* gene [78]. Because of the C57Bl/6 background (B6) of the MMP-9 knockout mice [67], $B6^{lpr/lpr}$ with the *lpr* mutation on a B6 background were used to avoid lengthy backcrossings from the MRL to the C57Bl/6 background (*cf.* Chapter 4). Whereas $MRL^{lpr/lpr}$ mice develop massive nonmalignant lymphoproliferation and extensive autoimmune tissue injury, in $B6^{lpr/lpr}$ mice the lupus-like syndrome is delayed and subdued [78].

Double negative (DN) T cells

In healthy persons and normal mice, DN T cells represent only 1% to 5% of peripheral lymphocytes and have regulatory functions. These 'regulatory DN T cells' are able to suppress the activity of $CD8^+$ and $CD4^+$ T cells in an antigen-specific manner by direct Fas/FasL-mediated killing [48]. Little, if any, defects in thymic negative selection are observed in the absence of a functional Fas pathway, ruling out defective T cell development as a major cause of lymphoproliferation [79]. Several genetic deficiencies lead to T cell lymphoproliferation, as was described for CTLA-4 deficient mice [80] and the T_{reg}-deficient scurfy mice [81], but only the lymphoproliferation induced by impairment of the Fas pathway (by homozygous *lpr* or *gld* mutations or other Fas mutations in patients with autoimmune lymphoproliferative syndrome (ALPS)) is dominated by DN T cells [78,82]. This was recently clarified by the finding that in the periphery, DN T cells die at exceptionally high rates by Fas-mediated apoptosis. Hence, in the absence of functional Fas, they progressively accumulate in peripheral lymphoid organs causing lymphadenopathy and splenomegaly [83]. The origin of DN T cells, however, remains controversial [48,79]. On the one

hand, DN T cells were suggested to develop from thymically derived $CD8^+$ T cells as upon activation *in vitro*, a fraction of $CD8^+$ T cells downregulates CD8 and acquires a pro-inflammatory cytokine production capacity [84]. On the other hand, CD8-deficient mice have fully functional regulatory DN T cells and these were suggested to mature extrathymically in organs such as bone marrow, appendix, or liver. In addition, DN T cells were found in the nasal-associated lymphoid tissue of normal and athymic nude mice [48]. Furthermore, it is not clear how these regulatory DN T cells switch from a suppressive to a pro-inflammatory phenotype in mice and patients with SLE, where the expanded DN T cell population provides help to autoreactive B cells, secretes pro-inflammatory cytokines and induces nephritis [46,49-51].

MMP-9 effects on leukocyte homeostasis

As detailed in Chapter 4, the presence of MMP-9 in $B6^{lpr/lpr}$ mice attenuates and delays the accumulation of DN T cells, the production of autoantibodies and the extent and severity of autoimmune tissue injury. Since MMP-9 controls dendritic cell stimulatory and alloreactive T cell responses [71,72], the question was raised whether MMP-9 may attenuate *lpr*-induced lymphoproliferation. Hence, future experiments will investigate whether MMP-9 deficieny enhances (DN) T and B cell proliferation *in vivo*. Alternatively, or on top of enhanced proliferation, lack of MMP-9 may result in an additional apoptosis defect, as was described in zymosan peritonitis [85] and as suggested by many pro-apoptotic cleavages of intracellular substrates (*cf.* Discussion Part I). Analysis of lymphocyte apoptosis in $B6^{lpr/lpr}$.MMP-$9^{-/-}$ and $B6^{lpr/lpr}$.MMP-$9^{+/+}$ mice will shed light on MMP-9-mediated regulation of apoptosis in systemic autoimmunity. Finally, since MMP-9 modulates cytokines and chemokines [61,62], as well as many membrane-associated molecules (*cf.* Chapter 1), it may be interesting to test whether cytokines such as BAFF and IL-10, and their respective receptors BAFF-R and IL-10R, may be modulated by MMP-9. In addition, MMP-9 mediates ectodomain cleavage of RAGE [86]. Since soluble RAGE was shown to inhibit activation of autoreactive B cells by chromatin ICs [87], measurement of soluble RAGE in $B6^{lpr/lpr}$ mice with or without MMP-9 deficiency may reveal *in vivo* proteolysis and the concomitant effects of RAGE shedding on systemic autoimmunity.

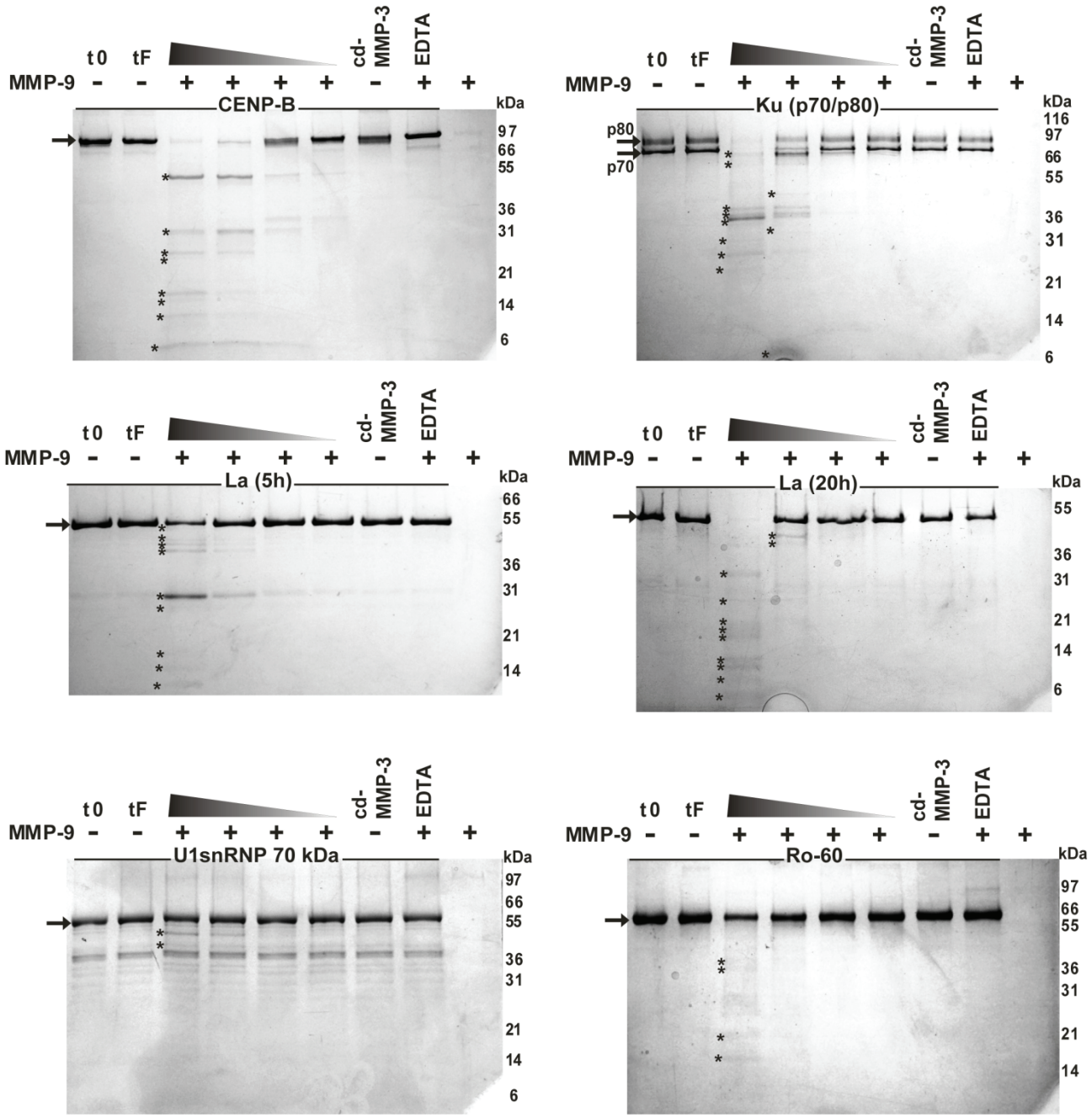


Figure 1. Degradation of SLE autoantigens by MMP-9 *in vitro*. The autoantigens (100 µg/mL) were incubated for 5h (and/or 20h for Ro-60 and La) in the absence (tF, -) or presence (+) of various concentrations of activated MMP-9 (200 nM to 1.6 nM, decreasing from lane 3 to 6) and analysed by SDS-PAGE. Control incubations included incubation with activated MMP-9 (200 nM) supplemented with the metalloprotease inhibitor EDTA (20 mM) and incubation with the catalytical domain of MMP-3 (cd-MMP-3, 2 nM), used to activate MMP-9. In addition, the highest concentration of MMP-9 was also incubated separately as a control for non-substrate fragment bands (lane 9). Visualization of the intact substrates (arrows) before incubation is shown under t0. Fragments generated by MMP-9 are indicated with asterisks (*). Apparent molecular masses are shown next to the gels in kDa. Rib. Protein P0, ribosomal protein P0; CENP-B, centromere protein B; U1snRNP, U1 small nuclear ribonucleoprotein.

MMP-9 and systemic autoantigen cleavage

MMP-9 may also control lymphoproliferation and autoantibody production by the proteolysis of systemic autoantigens. In systemic autoimmunity, most autoantibodies are directed to nucleic acid-binding proteins that are part of large intranuclear complexes such as nucleosomes, centromeres, small nuclear (sn) and nucleolar (sno) ribonucleoproteins (RNPs) or heterogenous nuclear (hn) RNPs, whereas other autoantibodies target particles that are temporarily located in the cytoplasm such as Ro RNPs, tRNA synthetase complexes and ribosomes. For example, the U1snRNP complex is a major component of the spliceosome and consists of multiple proteins, including the seven Sm core proteins (B and its splice variant B', D1, D2, D3, E, F, G) and the U1snRNP-specific proteins (U1snRNP 70 kDa, U1snRNP A and U1snRNP C) [9]. Many SLE autoantigens were found to be (candidate) substrates of MMP-9 by one-dimensional (*cf.* Chapter 2) and multidimensional (*cf.* Chapter 3) degradomics approaches. In addition, MMP-9 also cleaves major SLE autoantigens, such as the diagnostic Sm antigen B/B', Ro-52 (Sjögren's syndrome antigen A or SSA), ribosomal protein P0 (*cf.* Chapter 4), Sm antigen D3 (*cf.* Chapter 3) and La antigen (SSB) (Figure 1). Anti-Ro and anti-La antibodies are used for diagnosis in the absence of anti-double stranded(ds) DNA antibodies and have a high sensitivity and specificity for subacute cutaneous lupus (SCLE) and neonatal lupus erythematosus (NLE) [88]. Additional components of the Ro/La RNP complexes are nucleolin and calreticulin, which are also cleaved by MMP-9 (*cf.* Chapter 3) and other MMPs (*cf.* Discussion Part 1), respectively. The presence of anti-ribosomal protein P antibodies is associated with lupus cerebritis and psychosis [88]. Centromere protein B (CENP-B) is the immunodominant antigen of anti-centromere antibodies, which have a lower frequency in SLE but are often connected to the development of Raynaud's phenomenon and pulmonary hypertension [9].

Although some autoantigens were cleaved by MMP-9 with low efficiency (e.g. Ro-60 and U1snRNP 70 kDa), most autoantigens were cleaved fast and extensively (Figure 1). For example, digestion of CENP-B and Ku (p70/p80) resulted in a ladder pattern, much alike the degradation of CAP1 (*cf.* Chapter 2); actin and tubulin (*cf.* Chapter 3); and U1snRNP A and ribosomal protein P0 (*cf.* Chapter 4). In addition, up till now, we did not find an autoantigen that was not cleaved at all. Hence, cleavage of intracellular proteins by MMP-9 may be considered as predictive for their autoantigen status, as was suggested for caspases and granzyme B *(cf.* Discussion Part 1) [53].

MMP-9 and clearance of immunodominant epitopes

Many autoantibodies targeted at MMP-9 substrates have a high prevalence in SLE and are correlated with disease activity [8,9]. Hence, regarding the extent of SLE autoantigen cleavage, it was tempting to speculate that destruction of immunodominant epitopes for T and B cells contributes to the protective effects of MMP-9 in *lpr*-induced systemic autoimmunity. These cryptic epitopes are normally hidden inside cells, but when released into the extracellular milieu, their elimination by proteolysis may be crucial to maintain tolerance after (extensive) cell death. In $B6^{lpr/lpr}$ mice, the accumulated DN T cells will eventually undergo secondary necrosis and provide a continous pool of autoimmune stimuli, which may be supplemented with necrotic material from autoimmune tissue lesions. Therefore, the absence of MMP-9-mediated proteolysis may result in an additional clearance deficiency, which accelerates the onset of systemic autoimmunity and promotes its propagation (*cf.* Introduction).

In a first attempt to corroborate this, we analyzed autoantibody responses against two abundant intracellular MMP-9 substrates, actin and tubulin (*cf.* Chapter 4). Anti-actin and anti-tubulin IgG titers were indeed higher in $B6^{lpr/lpr}$.MMP-$9^{-/-}$ mice compared with $B6^{lpr/lpr}$.MMP-$9^{+/+}$ mice, but this may be a general consequence of enhanced systemic autoimmunity and epitope spreading to multiple autoantigens. A more controlled approach to verify the effect of MMP-9-mediated proteolysis on B cell epitopes was to compare recognition of intact or cleaved autoantigens by autoantibodies from SLE mice and patients using competitive ELISA (*cf.* Chapter 4). In this way, it became clear that proteolysis of the autoantigens ribosomal protein P0 and U1snRNP A by MMP-9 strongly disturbed autoantibody binding, confirming a destruction of B cell epitopes by MMP-9 in these autoantigens. Future experiments with multiple autoantigenic substrates of MMP-9 (and non-substrates) may reveal if clearance of B cell epitopes is a general effect of MMP-9-mediated proteolysis. Using plasma samples from $B6^{lpr/lpr}$.MMP-$9^{-/-}$ mice and $B6^{lpr/lpr}$.MMP-$9^{+/+}$ mice, the relative effects or proteolysis on conformational *vs.* linear B cell epitopes may be assessed by a comparison of Western Blot analysis (for linear epitopes) and immunoprecipitation followed by Western blot analysis (for conformational epitopes). To analyze the elimination (or formation) of autoreactive T cell epitopes, leukocytes of $B6^{lpr/lpr}$ mice and SLE patients may be stimulated to proliferate with intact and degraded autoantigens.

However, since cell death results in the release of the entire cellular proteome, the total immunogenic or tolerogenic effect of proteolysis on the immune system is the sum of the destruction and generation of immunodominant epitopes within each individual cellular antigen (and by the complete pool of available proteases). To investigate whether proteolysis by MMP-9 decreases or enhances the immunogenicity of the total intracellular protein pool, mice were immunized twice with THP-1 cytosol in complete Freund's adjuvant (CFA) (Figure 2). Lymph node T cell proliferation was compared after stimulation with MMP-9-cleaved *vs.* intact THP-1 cytosol. Interestingly, a significantly lower T cell proliferation was observed after stimulation with cleaved *vs.* intact intracellular proteins in the presence of antigen-presenting cells. This suggests that the net proteolytic effect of MMP-9 results in the elimination of immunodominant T cell epitopes. To confirm this *in vivo,* mice will be immunized with intact or cleaved cytosol.

Assessment of T and B cell proliferation *in vivo* and comparison of autoantibody titers may be another way of evaluating the effects of proteolysis on cellular and humoral autoimmune responses.

Many of the cleaved SLE autoantigens are shared by other systemic autoimmune diseases, for example Ro/La in Sjögren's syndrome, U1snRNP in mixed connective tissue disease, CENP-B in systemic sclerosis, Jo-1 in polymyositis/dermatomyositis and Ku (p70/p80) in a variety of connective tissue disorders [88]. In addition, autoantigenic substrates are shared by multiple MMPs (*cf.* Discussion Part 1), suggesting that clearance of systemic autoantigens may be a common function of MMPs.

Finally, as clearance deficiencies only trigger systemic autoimmunity on a susceptible background [27], it would be interesting to investigate whether the lack of MMP-9 is permissive for tolerance breaking after induction of extensive necrosis, which may be mimicked by applying a burn injury model to MMP-9 knockout and C57Bl/6 wildtype mice [42].

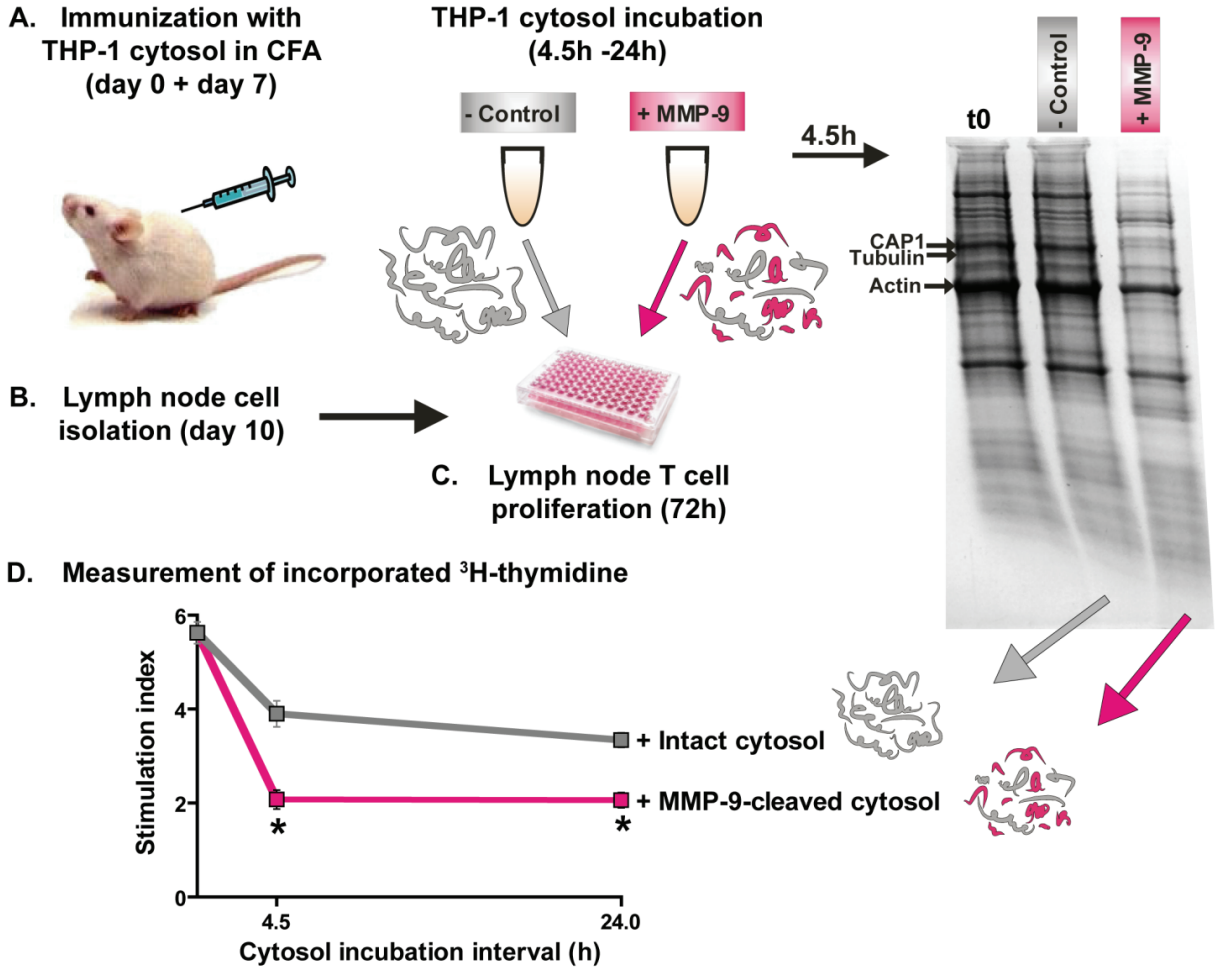


Figure 2. MMP-9-mediated proteolysis decreases general T cell responses to intracellular autoantigens. (A) NMRI mice were immunized subcutaneously with THP-1 cytosol (1 mg) in complete Freund's adjuvant (CFA) and boosted on day 7 with THP-1 cytosol in CFA (1 mg). (B) Lymph node cells were isolated on day 10 and (C) stimulated to proliferate with THP-1 cytosol that was previously incubated in the absence (– control) or presence (+ MMP-9) of activated MMP-9 (100 nM) for 4.5h and 24h, and with THP-1 cytosol that was not incubated (t0). Cytosol cleavage was verified by SDS-PAGE analysis. (D) Proliferation was assessed by measuring incorporated ^{3}H-thymidine (after 18h). *, $P < 0.05$, Mann-Whitney *U* test (n=6). CAP1, adenylyl cyclase-associated protein-1.

MMP-9 in the circulation of SLE patients

In accordance with the exacerbation of *lpr*-induced systemic autoimmunity in the absence of MMP-9, various studies report lower MMP-9 levels in the circulation of SLE patients compared with healthy controls and in SLE patients with active disease compared with inactive disease (Table 1). However, other studies report no differences or increased levels of MMP-9 in SLE patients. These controversial results may be partly explained by the heterogeneity of the SLE patient population, with damage to different organ systems, and with both relapsing patients with active disease and patients in remission with inactive disease.

Table 1. Serum MMP-9 levels/activities in SLE patients compared with healthy controls.

Study	Method[1]	Conclusions	SLE *vs.* control[2]
Faber-Elmann *et al.*, 2002 [89]	Gel zymo, activity assay	Increased serum MMP-9 activities in SLE patients *vs.* healthy controls. MMP-9 levels only correlated with SLE activity index in male patients.	↑
Makowski and Ramsby, 2003 [90]	Gel zymo	Inverse correlation of serum MMP-9 levels with anti-dsDNA antibody titers.	↓
Ainiala *et al.*, 2004 [91]	ELISA	No significant difference in serum MMP-9 levels in SLE patients and controls and no correlation with SLE activity index. Significantly elevated serum MMP-9 levels in NPSLE patients *vs.* non-NPSLE patients.	↔
Liu et *al.*, 2004 [92]	ELISA	Decreased MMP-9 serum levels in SLE patients *vs*. healthy controls, in patients with active disease *vs.* inactive disease and in patients with renal damage *vs.* without renal damage.	↓
Robak *et al.*, 2006 [93]	Activity assay	Decreased total and activated MMP-9 serum levels in SLE patients *vs.* healthy controls and in patients with active disease vs. inactive disease. No correlation with SLE activity index.	↓
Chang *et al.*, 2008 [94]	Gel zymo, ELISA	Increased serum MMP-9 levels in SLE patients *vs.* healthy controls	↑
Rho *et al.*, 2008 [95]	ELISA	No significant difference in serum MMP-9 levels in SLE patients and controls.	↔
Hou and Zhang, 2008 [96]	ELISA	Decreased MMP-9 serum levels in SLE patients *vs.* healthy controls. No correlation of MMP-9 levels with SLE activity index.	↓
Jiang *et al.*, 2009 [97]	ELISA	No significant differences in MMP-9 and MMP-9/TIMP-1 ratios between non-nephritic SLE patients and healthy controls. Increased serum MMP-9 levels and MMP-9/TIMP-1 ratios in nephritic SLE patients *vs.* non-nephritic SLE patients and healthy controls. Negative correlation between serum MMP-9/TIMP-1 ratios and anti-dsDNA antibody titers.	↔
Lesiak *et al.*, 2010 [98]	ELISA	Increased MMP-9 and TIMP-1 levels in SLE patients *vs.* healthy controls. Decreased MMP-9 and increased TIMP-1 levels after hydroxychloroquine treatment.	↑

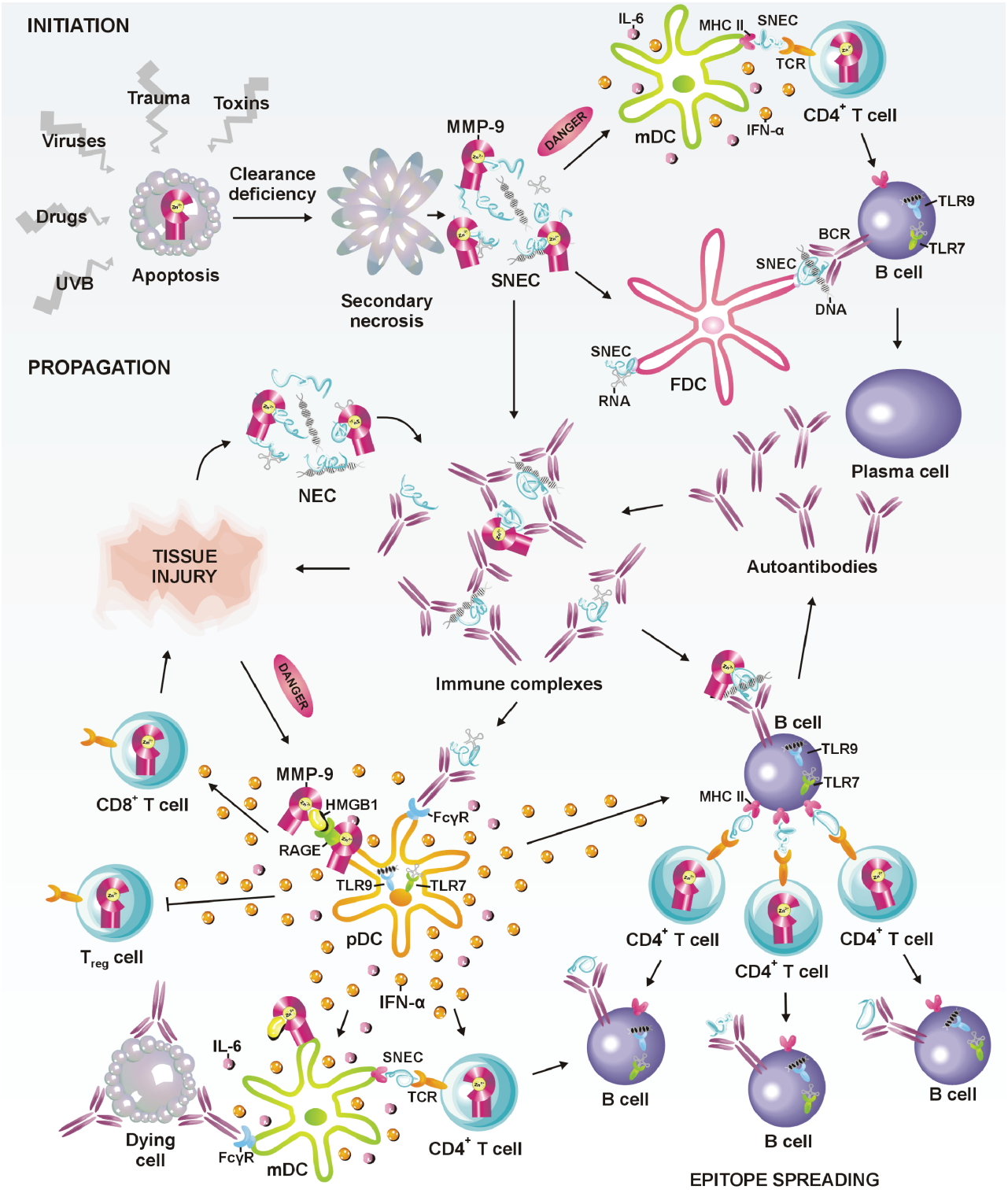
[1]Methods used voor the measurement of MMP-9 levels or activities: **Activity assay** based on affinity capture and cleavage of a quenched fluorescent peptide; **Gel zymo**, gel zymography or substrate zymography; **ELISA**, enzyme-linked immunosorbent assay.

[2]Comparison of MMP-9 levels in SLE patients *vs.* healthy controls: ↑, increased in SLE *vs.* control; ↔, no differences between SLE and control; ↓decreased levels in SLE *vs.* control.

In addition, higher MMP-9 levels may be a consequence of higher production in chronically inflamed organs, which is underscored by higher MMP-9 levels in patients with neuropsychiatric SLE (NPSLE) [91] and lupus nephritis [97]. However, in the case of lupus nephritis, decreased MMP-9 levels in nephritic *vs.* non-nephritic patients have also been reported, whereas we found increased levels of pro- and activated MMP-9 in the urines of patients with indications of renal failure (*cf.* Chapter 2). The main complication with these studies is the fact that MMP-9 levels have been measured in serum instead of in plasma. Serum is not an ideal source for the analytical determination of circulating MMP-9 levels as during coagulation/fibrinolysis activated platelets and leukocytes, such as degranulating neutrophils, will result in artefactually high MMP-9 levels, which is even more pronounced for serum samples collected in tubes with clot activators [99]. Since peripheral blood mononuclear cells (PBMCs) from SLE patients were found to secrete higher MMP-9 levels *in vitro* than PBMCs from control patients [96,100], which probably reflects their increased activation status, secretion during clotting will readily contribute to the higher serum levels reported in several SLE studies. During the present doctoral research, fresh frozen plasma samples from patients with rheumatic diseases such as SLE, Sjögren's syndrome, vasculitis, systemic sclerosis and polymyositis were collected by immediate centrifugation and freezing on dry ice, followed by storage at -70°C. Preliminary data show decreased plasma MMP-9/MMP-2 in systemic autoimmune patients compared with healthy controls, but this needs to be confirmed with more healthy control samples in order to reach final conclusions. In addition, MMP-9/TIMP-1 ratios should be determined in order to evaluate the potential *in vivo* activity of MMP-9. Hence, MMP-9 levels may be decreased in SLE patients before the appearance of clinical symptoms and during onset and amplification stages, contributing to clearance deficiencies in SLE. However, autoimmune organ damage may lead to enhanced production of MMP-9 in inflamed tissues, where it might contribute to clearance of intracellular contents released by necrotic lesions. In accordance with this hypothesis, increased MMP-9 activities were detected only in later stages of experimental SLE, when clinical manifestations were already observed [101].

Conclusion

The net protective effect exerted by MMP-9 in *lpr*-induced systemic autoimmunity may be based on multiple mechanisms, as summarized in Figure 3. Further elucidation of the individual mechanisms that contribute to the exacerbation of *lpr*-induced systemic autoimmunity in the absence of MMP-9 may have clinical significance. Indeed, since systemic autoantigen cleavage is a common feature of MMPs, the chronic use of general or broad-spectrum MMP inhibitors should not be recommended as it may trigger or exacerbate systemic autoimmunity in a susceptible host.



Discussion Figure 3. Protective roles of MMP-9 in the etiopathogenesis of SLE. MMP-9 may promote apoptosis by extracellular or intracellular cleavages (*cf.* Discussion Part 1). MMP-9 degrades many intracellular proteins and systemic autoantigens, and this may contribute to the immunogenic clearance of secondary necrotic cell-derived material (SNEC) by destruction of immunodominant epitopes for T and B cells (*cf.* Chapters 2 and 3). MMP-9 may contribute to leukocyte homeostasis by dampening (double negative) T cell proliferation (*cf.* Chapter 4). After upregulation by tissue injury and inflammation, MMP-9 may contribute to the removal of immunogenic necrotic cell-derived material (NEC) and immune complexes. In addition, MMP-9 degrades various danger signals or alarmins (*cf.* Chapter 3 and Discussion Part 1), such as HMGB1 and its receptor RAGE, possibly inactivating the proinflammatory danger signaling. Finally, proteolysis by MMP-9 may generate low doses of self-peptides, which promote tolerance by the induction of regulatory T cells (T_{regs}) and anti-inflammatory cytokines that suppress pathogenic T and B cells. BCR, B cell receptor; FcγR, Fcγ receptor; IL-6, interleukin-6; IFN-α, interferon-α; MHC II, major histocompatibility complex Class II; TCR, T cell receptor; TLR7/9, Toll-like receptor 7/9; UVB, ultraviolet B.

Future perspectives

The enhanced lymphadenopathy observed in $B6^{lpr/lpr}$.MMP-$9^{-/-}$ *vs.* $B6^{lpr/lpr}$.MMP-$9^{+/+}$ mice may stem from increased cell proliferation or additional apoptotic defects in the absence of MMP-9. Incorporation of Edu (5-ethynyl-2′-deoxyuridine) will be combined with flow cytometry analysis to compare proliferation and apoptosis of different lymphocyte subsets in both groups of mice. Shedding and inactivation of proliferation-inducing membrane molecules by MMP-9 may be at the basis of a proliferation-suppressive effect. Hence, proteolysis of such membrane-associated candidates will be assessed in $B6^{lpr/lpr}$.MMP-$9^{-/-}$ *vs.* $B6^{lpr/lpr}$.MMP-$9^{+/+}$ mice.

MMP-9 may also control lymphoproliferation and autoantibody production by the clearance of systemic autoantigen epitopes. Indeed, MMP-9-mediated degradation of systemic autoantigens such as ribosomal protein P0 and U1snRNP A largely abolishes recognition by autoantibodies from $B6^{lpr/lpr}$ mice as well as from SLE patients, as demonstrated with competitive ELISA experiments. These experiments may be corroborated with data from immunoprecipitations of cleaved and intact autoantigens with plasma samples from SLE mice and patients. In addition, to investigate if proteolysis has general epitope-degrading effects in multiple autoantigens, differentially labeled cleaved and uncleaved cytosol samples may be subjected to immunoprecipitation and further analyzed by 1-dimensional or 2-dimensional SDS-PAGE analysis. The relative effects or proteolysis on conformational *vs.* linear B cell epitopes may be assessed by a comparison of Western Blot analysis (for linear epitopes) and immunoprecipitation followed by Western blot analysis (for conformational epitopes). To analyze the elimination (or formation) of autoreactive T cell epitopes, leukocytes of $B6^{lpr/lpr}$ mice and SLE patients may be stimulated to proliferate with intact and degraded autoantigens both *ex vivo* and *in vivo*.

In a more biochemical approach, the cleavage sites of MMP-9 in systemic autoantigens will be determined by Edman degradation and LC-MS/MS. The identified cleavage sites may then be compared with known immunodominant epitopes of systemic autoantigens [9], in order to determine if these are released or destroyed by MMP-9-mediated proteolysis.

Since proteolysis by MMP-9 abolishes autoantibody recognition of systemic autoantigens such as ribosomal protein P0 and U1snRNP A, immune complexes (ICs) containing these autoantigens will be isolated by gel filtration chromatography and incubated with MMP-9 to assess whether proteolysis may dissociate these complexes. Alternatively, formation of ICs between isolated free autoantibodies and cleaved or intact autoantigens will be compared. These *ex vivo* experiments may be complemented *in vivo* by injecting $B6^{lpr/lpr}$.MMP-$9^{-/-}$ mice (having high autoantibody titers) with high doses of activated MMP-9 for one or two weeks, after which autoantibody and IC concentrations should be reassessed to evaluate the IC clearance potential of MMP-9 *in vivo*.

Alternatively, B6.MMP-$9^{-/-}$ and B6 control mice will be immunized various times with cleaved or intact autoantigen (e.g. ribsomal protein P0) in CFA and the development of autoantibody titers will be compared. Since in all of these experiments cleavage by MMP-9 occurs *ex vivo*, an experiment will also be included in which B6.MMP-$9^{-/-}$ and B6 control mice are immunized only with intact autoantigen or cytosol in order for the proteolysis of the autoantigen(s) to take place *in*

vivo. Autoantibody titers, epitope spreading and SLE symptoms such as proteinuria will be assessed during various months. In order to confirm *in vivo* cleavage of systemic autoantigens, one of the mentioned degradomics approaches (*cf*. Discussion Part 1) may be applied to organ extracts or plasma samples of $B6^{lpr/lpr}$.MMP-$9^{-/-}$ *vs.* $B6^{lpr/lpr}$.MMP-$9^{+/+}$ mice, as well as immunized B6.MMP-$9^{-/-}$ and B6 control mice.

Finally, the application of a burn injury model to MMP-9 knockout and B6 wildtype mice may be a way of testing whether lack of MMP-9 may result in a clearance deficiency that results in the break of tolerance to intracellular antigens.

Regarding the levels of MMP-9 in SLE patients and in patients with other rheumatic diseases, a group of control samples from healthy donors will be collected to compare the plasma and urinary MMP-9/MMP-2 levels and MMP-9/TIMP-1 ratios, and the correlations between various autoantibody titers, proteinuria and MMP-9 levels.

SUMMARY

Matrix metalloproteinases (MMPs) constitute a family of 24 different neutral endopeptidases in human. These proteases intervene in physiological processes such as reproduction, development, immunity and tissue repair. However, dysregulation of MMP activity has multiple pathogenic effects in cancer, inflammation, vascular diseases, neurodegenerative disorders and autoimmune diseases. Gelatinase B/MMP-9 is secreted by a variety of immune and cancer cells, major producers being neutrophils and macrophages at sites of inflammation. MMP-9 was previously shown to generate immunodominant T cell epitopes from myelin basic protein and αB-crystallin in multiple sclerosis, from collagen type II in rheumatoid arthritis, and from insulin in type I diabetes. This may explain the pathogenic effect of MMP-9 and why MMP-9-deficient mice are less susceptible to develop disease symptoms in these organ-specific autoimmune diseases. Hence, the question was raised whether MMP-9 may proteolyse systemic autoantigens and how this may affect the development of systemic autoimmunity.

Whereas organ-specific autoimmune diseases target a limited number of autoantigens that are mostly confined to the affected organ, in systemic autoimmunity the autoantigen spectrum is generally very broad and mainly comprises ubiquitous intracellular proteins. The prototypic autoimmune condition, systemic lupus erythematosus (SLE), is characterized by high titers of autoantibodies against more than a hundred different intracellular and intranuclear autoantigens. The formation and deposition of ICs causes inflammation and tissue injury throughout the body, affecting the skin, kidneys, joints, lungs, central nervous system and blood vessels. In SLE, enhanced apoptosis may be triggered by genetic and environmental factors (e.g. viral infection, drugs, toxins). In addition, various mechanisms for the clearance of dying cells are impaired. As a consequence, the overload of apoptotic cells progresses to secondary necrosis and intracellular proteins are released into the extracellular milieu, where they may be accessed by MMPs and other proteases. Therefore, a first rationale for the study of MMP-9 in systemic autoimmunity was to investigate whether MMP-9 might cleave SLE autoantigens.

To define whether MMP-9 is able to proteolyze systemic autoantigens, we started an unbiased, systematic characterization of the intracellular substrate repertoire or 'degradome' of MMP-9 using a one-dimensional degradomics approach. To mimick the necrotic release of intracellular proteins in SLE, we used a model system consisting of dying human monocytic THP-1 cells, supplemented or not with activated MMP-9. In this way, we identified adenylyl cyclase-associated protein-1 or CAP1 as a novel and highly efficient substrate of MMP-9. CAP1 is a cytoskeletal protein that was identified as an autoantigen in SLE and rheumatoid arthritis. Moreover, we detected intact CAP1 in the urines of patients with systemic autoimmune diseases such as SLE, Sjögren's syndrome and vasculitis, as well as in healthy control urines. Whereas no active MMP-9 was detected in urines of healthy controls, all urine samples of patients with clinical parameters of renal failure contained activated MMP-9. In addition, in some patient urines, an inverse relation was observed between the levels of intact CAP1 and activated MMP-9, suggesting CAP1 degradation *in vivo*.

Identification of additional substrates by this one-dimensional degradomics approach was hampered by the complexity of the cellular protein pool. In order to proceed to higher resolution identification of intracellular MMP-9 substrates, a second aim was to develop a straightforward and inexpensive multidimensional degradomics platform. This multidimensional degradomics technology reduces the sample complexity by first separating the proteins according to their net charge or isoelectric point using ion exchange chromatography, followed by a second dimension of separation by molecular weight on SDS-PAGE and by centrifugal filtration steps. Application of this multidimensional degradomics technology on THP-1 cytosol resulted in the isolation of 100-200 MMP-9 candidate substrates, of which about 70 were identified by tandem mass spectrometry (MS/MS). Of interest, about 2/3 of these candidate substrates were autoantigens described in one or multiple autoimmune conditions and in cancer. In addition, about 40% of the substrates were intracellular matrix proteins, such as actin and tubulin. These results showed that systemic autoantigen proteolysis might indeed be a function of MMP-9, but also suggested that proteolysis by MMP-9 may be required to clear the toxic and immunogenic burdens of intracellular (matrix) proteins and systemic autoantigens released after extensive necrosis.

To address the *in vivo* role of MMP-9 in systemic autoimmunity, a third and long-term aim was to generate mice that lack MMP-9 and have the *lpr* (lymphoproliferative) loss-of-function mutation in the apoptosis-inducing receptor Fas. C57Bl/6 mice without functional Fas ($B6^{lpr/lpr}$ mice) develop moderate lymphoproliferation and late-onset systemic autoimmunity with little immunopathology. However, the additional genetic knockout of MMP-9 in $B6^{lpr/lpr}$ mice resulted in greatly amplified and earlier onset lymphoproliferative disease with enhanced lymphadenopathy and splenomegaly, and significantly reduced survival compared with single Fas deficiency. In addition, the absence of MMP-9 in $B6^{lpr/lpr}$ mice resulted in increased autoantibody production against multiple autoantigens and more pronounced autoimmune tissue injury. Since 'intact' autoantigens seemed to be better stimuli for autoantibody production, suppression of *lpr*-induced systemic autoimmunity may be a consequence of MMP-9-mediated clearance of immunodominant T and B cell epitopes in autoantigenic substrates. Hence, deficiency of MMP-9-mediated proteolysis may engender an additional clearance deficiency, which accelerates the onset of systemic autoimmunity and promotes its amplification.

In conclusion, MMP-9 degrades a whole spectrum of intracellular (matrix) proteins and systemic autoantigens. Clearance of intracellular proteins may be of crucial importance to preserve immune tolerance after acute or chronic necrosis, as observed in SLE. Along this line, MMP-9 was identified as a protective factor in an *in vivo* model of systemic autoimmunity. Our preclinical studies have medical implications. Some recent studies suggest the use of MMP inhibitors for SLE and other systemic autoimmune disease. Our studies indicate that MMP inhibition for the treatment of SLE needs to be studied with sufficient reservation as it may trigger or exacerbate systemic autoimmunity in a susceptible host.

SAMENVATTING

Bij de mens omvat de familie van de matrix metalloproteïnasen (MMPs) 24 verschillende neutrale endopeptidasen. Deze proteasen komen tussen in fysiologische processen zoals reproductie, ontwikkeling, immuniteit en weefselherstel. Ontregeling van MMP activiteit heeft echter pathogene effecten bij kanker, inflammatie, vasculaire ziekten, neurodegeneratieve aandoeningen en auto-immuunziekten. Gelatinase B/MMP-9 wordt vooral gesecreteerd door macrofagen en neutrofiele granulocyten tijdens inflammatie, maar wordt ook geproduceerd door veel andere immuun- en kankercellen. Proteolyse van auto-antigenen door MMP-9 genereert immunodominante restepitopen voor auto-reactieve T cellen. Dit werd reeds aangetoond voor de klieving van collageen type II in reumatoïde artritis, van 'myelin basic protein' en αB-crystalline in multiple sclerose, en van insuline in diabetes type I. Bovendien bleken MMP-9-deficiënte muizen minder gevoelig voor het ontwikkelen van symptomen in diermodellen van deze orgaanspecifieke auto-immuunziekten. Bijgevolg werd de vraag gesteld of MMP-9 ook systemische auto-antigenen kan klieven en hoe dit de ontwikkeling van systemische auto-immuniteit zou beïnvloeden.

Orgaan-specifieke auto-immuniteit is meestal gericht tegen een beperkt aantal auto-antigenen die voorkomen in het aangetaste orgaan. Systemische auto-immuunziekten worden daarentegen gekenmerkt door een breed spectrum aan auto-antigenen, vooral bestaande uit veel voorkomende intracellulaire eiwitten. Systemische lupus erythematosus of SLE is het type-voorbeeld van een systemische auto-immuunziekte en wordt gekenmerkt door hoge titers van auto-antistoffen tegen meer dan 100 verschillende intracellulaire and intranucleaire auto-antigenen. De vorming en afzetting van immuuncomplexen veroorzaakt inflammatie en weefselschade over het hele lichaam en tast vooral huid, nieren, gewrichten, longen, centraal zenuwstelsel en bloedvaten aan. In SLE veroorzaken genetische en omgevingsfactoren (bv. virale infecties, medicatie, toxines) een toename van apoptose of geprogrammeerde celdood. Bovendien zijn verschillende mechanismen voor het opruimen van apoptotische cellen verstoord. Bijgevolg zal de overmaat aan apoptotische cellen leiden tot secundaire necrose en openbarsten. Hierdoor worden intracellulaire eiwitten vrijgesteld in het extracellulair milieu, waar ze kunnen gemoduleerd worden door MMPs en andere proteasen, hetgeen een extra argument vormt voor de studie van MMP-9 in systemische auto-immuniteit.

Om te bepalen of MMP-9 in staat is om systemische auto-antigenen te klieven, werd een systematische karakterisatie van het intracellulaire substraatrepertoire of 'degradoom' van MMP-9 opgestart aan de hand van een 1-dimensionele 'degradomics' methode. Om het necrotisch vrijstellen van intracellulaire eiwitten in SLE na te bootsen, werd een modelsysteem gebruikt van necrotische humane monocytische THP-1 cellen, met of zonder toevoeging van geactiveerd MMP-9. Op deze manier werd 'adenylyl cyclase-associated protein-1' of 'CAP1' geïdentificeerd als een nieuw en uitzonderlijk efficiënt substraat van MMP-9. CAP1 is een cytoskeleteiwit en een auto-antigeen in reumatoïde artritis en SLE. Bovendien, detecteerden we intact CAP1 in urines van patiënten met systemische auto-imuunziekten zoals SLE, Sjögrens syndroom en vasculitis, en in urines van gezonde personen. Verder werden geactiveerde vormen van MMP-9 teruggevonden in urinestalen van patiënten met klinische parameters van nierfalen, terwijl in urines van personen

zonder nierziekte geen actief MMP-9 aanwezig was. Bovendien, werd in sommige urines van patiënten een omgekeerde relatie teruggevonden tussen de niveaus van intact CAP1 en van geactiveerd MMP-9, hetgeen suggereert dat CAP1 *in vivo* wordt afgebroken.

Identificatie van bijkomende substraten was door de complexiteit van het eiwitmengsel niet mogelijk met een 1-dimensionele aanpak. Om op grotere schaal intracellulaire MMP-9 substraten te kunnen identificeren, werd eenvoudige en goedkope multidimensionele degradomics technologie ontwikkeld. Deze multidimensionele methoden reduceren de complexiteit van het proteïnemengsel door de eiwitten eerst te scheiden volgens hun netto-lading of isoëlektrisch punt met behulp van ionenuitwisselingschromatografie en vervolgens in een tweede dimensie volgens moleculair gewicht met behulp van SDS-PAGE en centrifugale filtratie. Toepassing van de multidimensionele degradomics technologie op THP-1 cytosol leidde tot de isolatie en visualisatie van ongeveer 200 kandidaatsubstraten waarvan een 70-tal geïdentificeerd werden m.b.v. tandem massaspectrometrie (MS/MS). Ongeveer 2/3 van deze kandidaatsubstraten was reeds bekend als auto-antigeen in verschillende auto-immuunziekten en kankers. Ook werden talrijke intracellulaire matrix eiwitten, zoals actine en tubuline, geïdentificeerd als nieuwe substraten van MMP-9. Deze resultaten toonden aan dat proteolyse van systemische auto-antigenen wel degelijk een functie van MMP-9 kan zijn. Bovendien leidden deze data tot de suggestie dat proteolyse door MMP-9 vereist zou kunnen zijn om de overmaat aan toxische, immunogene intracellulaire (matrix) eiwitten en systemische auto-antigenen op te ruimen die worden vrijgesteld na extensieve necrose.

Om de rol van MMP-9 in systemische auto-immuniteit *in vivo* te onderzoeken, werden muizen gegenereerd die genetisch deficiënt zijn in MMP-9 en met de inactiverende *lpr* (lymfoproliferatie) mutatie in de apoptose-inducerende receptor Fas. C57Bl/6 muizen zonder functionele Fas receptor ($B6^{lpr/lpr}$ muizen) ontwikkelen matige lymfoproliferatie en chronische systemische auto-immuniteit op latere leeftijd en met weinig immunopathologie. De additionele genetische knockout van MMP-9 in $B6^{lpr/lpr}$ muizen resulteerde echter in een versnelde en sterk toegenomen lymfoproliferatie met uitgesproken lymfadenopathie en splenomegalie, en significant gereduceerde overleving vergeleken met enkel deficiëntie van Fas. Bovendien leidde het ontbreken van MMP-9 in $B6^{lpr/lpr}$ muizen tot een verhoogde productie van auto-antistoffen tegen multipele auto-antigenen en tot meer uitgesproken auto-immune weefselschade. Vermits 'intacte' auto-antigenen betere stimuli bleken te zijn voor auto-antilichaamproductie, zou de onderdrukking van *lpr*-geïnduceerde systemische auto-immuniteit een gevolg kunnen zijn van de opruiming van immunodominante T- en B-cel epitopen in auto-antigenische substraten. Bijgevolg zou het ontbreken van MMP-9-gemedieerde proteolyse tot een bijkomend defect in opruiming kunnen leiden, hetgeen de ontwikkeling van systemische auto-immuniteit kan versnellen en bestaande auto-immuunreacties kan amplificeren.

We kunnen dus besluiten dat MMP-9 een uitgebreid spectrum aan intracellulaire (matrix) proteïnen en systemische auto-antigenen kan klieven. Opruiming van intracellulaire eiwitten zou cruciaal kunnen zijn om immuuntolerantie te behouden na acute of chronische necrose, zoals voorkomend bij SLE. Bovendien werd MMP-9 geïdentificeerd als een beschermende factor in een *in vivo* model van systemische auto-immuniteit. Deze pre-klinische studies hebben medische implicaties.

Verschillende studies stellen immers voor om MMP remmers te gebruiken in SLE en andere systemische auto-immuunziekten. Onze data suggereren echter dat men voorzichtig moet omspringen met de studie van MMP inhibitie bij SLE, aangezien hierdoor systemische auto-immuunreacties kunnen ontstaan of geamplificeerd worden in patiënten met een ongunstige genetische achtergrond.

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CURRICULUM VITAE

PERSONALIA

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EDUCATION

- **UNIVERSITY**:

2005-2010 Doctoral training in Biomedical sciences
Option: **Immunology and microbiology**

Doctoral research: *The role of gelatinase B/MMP-9 in systemic autoimmunity*

2002-2005 Master in Bioscience Engineering ***Magna cum laude***
Major option: **Cell and gene biotechnology**
Minor option: **Food and health technology**

Master's Thesis: '*Het degradoom van gelatinase B/MMP-9: celgebonden en oplosbare substraten* in vitro *en* in vivo', Promotors: Prof. Dr. B. Goddeeris, Prof. Dr. G. Opdenakker.

1.09.03 – 1.02.04 *Erasmus exchange: Universidad Politécnica de Valencia, Spain*

1999-2002 Bachelor in Bioscience Engineering ***Cum laude***

- **SECONDARY SCHOOL**:

1993-1998 Latin-mathematics (8h) ***Summa cum laude***
Sint-Hubertuscollege, Neerpelt

ADDITIONAL CERTIFICATES

- **Laboratory Animal Science** (module I), KULeuven, 2005
- **Advanced Course in Laboratory Animal Science** (module II), KULeuven, 2006
- **Radioprotection ' Open sources and X-radiation'**, KULeuven, 2006
- **Applied Biostatistics**, KULeuven, 2009

SCIENTIFIC TRAINING

- **Truncus Communis Thema 4: Confocal microscopy: principles and applications** (Universiteit Hasselt, 3/02/06)

- **Two-dimensional Difference Gel Electrophoresis (2D-DIGE) Workshop**, CRP Gabriel Lipmann, Luxembourg, 9-11th of November, 2006.

- European Federation of Immunological Societies (EFIS) – European Journal of Immunology (EJI) Advanced Course on: **"The Role of B Cells in the Physiology and Pathology of the Immune System",** The Ruggero Ceppellini Advanced School of Immunology, Sorrento, Italy, 5-7th of November, 2009.

ORAL PRESENTATIONS

- **DEPARTMENTAL RESEARCH SEMINAR in the framework of the PhD training program**, Rega Institute for Medical Research, Leuven, Belgium, November 7, 2007. *'The role of gelatinase B/MMP-9 in systemic autoimmunity – from cell culture to patient sample'*

- **PROTEOMLUX 2008 – 'International conference on Proteomics in plants, microorganisms and environment'**, City of Luxembourg, Luxembourg, October 22nd, 2008. *'2-Dimensional degradomics as a powerful tool to identify novel gelatinase B/MMP-9 substrates',* **Bénédicte Cauwe**, Erik Martens, Paul Proost and Ghislain Opdenakker.

- **PROTEOMICS AND METABOLOMICS SYMPOSIUM organized by the Interfacultary Center for Proteomics and Metabolomics (ProMeta)**, Leuven, Belgium, September 11th, 2009. *'2-Dimensional degradomics as a powerful tool to identify novel gelatinase B/MMP-9 substrates',* **Bénédicte Cauwe**, Erik Martens, Paul Proost, Nele Berghmans, Chris Dillen and Ghislain Opdenakker.

- **'IMMUNOLOGY OF AUTOIMMUNITY 2009 MEETING'**, Leuven, Belgium, November 16th, 2009. '*Gelatinase B/MMP-9 cleaves intracellular substrates and thus decreases general T cell responses to (systemic auto)antigens'.* **Bénédicte Cauwe**, Erik Martens, Paul Proost, Nele Berghmans, Chris Dillen and Ghislain Opdenakker.

- **'AUTOIMMUNITY JOINT MEETINGS',** Leuven, Belgium, January 11th, 2010. '*The role of gelatinase B/MMP-9 in the development of lymphoproliferation and systemic autoimmunity in B6$^{lpr/lpr}$ mice.'* **Bénédicte Cauwe** *et al.*

- **LITERATURE SEMINAR in the framework of the PhD training program**, Rega Institute for Medical Research, Leuven, Belgium, April 13th, 2010*. Intracellular substrates – a novel dimension in the kaleidoscope of matrix metalloproteinase actions.*

POSTER PRESENTATIONS

- **Gordon Conference on Matrix Metalloproteinases - Signalling scissors in development and disease**, Switzerland, August 30 to September 4, 2009. *'Gelatinase B/MMP-9 cleaves cytosolic substrates and thus decreases general T cell responses to intracellular (matrix) proteins',* **Bénédicte Cauwe**, Erik Martens, Paul Proost, Nele Berghmans, Chris Dillen and Ghislain Opdenakker

- **2nd European Congress of Immunology (ECI)**, Berlin, Germany, September 13 to 16, 2009. *Gelatinase B/MMP-9 cleaves intracellular substrates and thus decreases general T cell responses to cytosol'*, **Bénédicte Cauwe**, Erik Martens, Paul Proost, Nele Berghmans, Chris Dillen and Ghislain Opdenakker.

- **EFIS-EJI meeting on: "The Role of B Cells in the Physiology and Pathology of the Immune System",** Sorrento, Italy, 5-7th of November, 2009. *Gelatinase B/MMP-9 cleaves intracellular substrates and thus decreases general T cell responses to cytosol'*, **Bénédicte Cauwe**, Erik Martens, Paul Proost, Nele Berghmans, Chris Dillen and Ghislain Opdenakker.

AWARDS AND GRANTS

- **STUDENT POSTER AWARD** at the **Gordon Conference on Matrix Metalloproteinases - Signalling scissors in development and disease**, Switzerland, August 30 to September 4, 2009.

- **TRAVEL GRANT of the Belgian Immunological Society (BIS)** to attend the **2nd European Congress of Immunology (ECI)**, Berlin, Germany, September 13 to 16, 2009.

- **YOUNG INVESTIGATOR AWARD for the best scientific presentation at the 'IMMUNOLOGY OF AUTOIMMUNITY 2009 MEETING'**, Leuven, Belgium, November 16th, 2009.

INTERNATIONAL PUBLICATIONS

- **Cauwe B**, Van den Steen PE, Opdenakker G. *The biochemical, biological, and pathological kaleidoscope of cell surface substrates processed by matrix metalloproteinases.* ***Crit Rev Biochem Mol Biol.*** (2007). 42(3):113-185.

- Descamps FJ, Kangave D, **Cauwe B**, Martens E, Geboes K, Abu El-Asrar A, Opdenakker G. *Interphotoreceptor retinoid binding protein as biomarker in systemic autoimmunity with eye inflictions.* ***J Cell Mol Med.*** (2008). 12(6A):2449-2456.

- Maes P, Clement J, **Cauwe B**, Bonnet V, Keyaerts E, Robert A, Van Ranst M. *Truncated recombinant puumala virus nucleocapsid proteins protect mice against challenge in vivo*. ***Viral Immunol.*** (2008). 21(1):49-60.

- **Cauwe B**, Martens E, Van den Steen PE, Proost P, Van Aelst I, Blockmans D, Opdenakker G. *Adenylyl cyclase-associated protein-1/CAP1 as a biological target substrate of gelatinase B/MMP-9.* ***Exp Cell Res.*** (2008). 314(15):2739-2749.

- **Cauwe B**, Martens E, Proost P, Opdenakker G. *Multidimensional degradomics identifies systemic autoantigens and intracellular matrix proteins as novel gelatinase B/MMP-9 substrates.* ***Integr. Biol.*** (2009). **1**(5-6) : 404 – 426.

- **Cauwe B** and Opdenakker G. *Intracellular substrate cleavage: a novel dimension in the biochemistry, biology and pathology of matrix metalloproteinases.* ***Crit Rev Biochem Mol Biol.*** (2007). In press.

- **Cauwe B**, Martens E, Sagaert X, Dillen C, Geurts N, Li S, Mertens J, Thijs G, Van den Steen PE, Heremans H, De Vos R, Blockmans D, Arnold B and Opdenakker G. *Gelatinase B/MMP-9 suppresses lpr-induced lymphoproliferation and lupus-like systemic autoimmune disease.* Submitted for publication.